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Remarks:
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Method and kit for detecting a sequence variation in the human TGF-beta 1 gene

(57) A method of detecting the presence or absence of the 713-8delC sequence variation in the human TGF-β1 gene is described, which method comprises provision of a sample comprising the TGF-β1 gene to be tested, or a relevant fragment thereof, subjecting the sample to a PCR amplification process using as upstream primer an oligonucleotide comprising a 15-100 nucleotides long section selected coherently from the following nucleotide sequence:

```
                 *
3' G TTT CGT ACC AAG TGA TGG CCG GCG
     GCT CCA CTG GAC CGG TGG TAA GTA
     CCG TAC TTG GCC GGA AAG GAC GAA
     GAG TAC CGG TGG GGC GAC CTC TCC
     CGG GTC GTA GAC  5'
```

such as to comprise the bold A* (the A marked with an asterisk) nucleotide, and as downstream primer a 15-100 nucleotides long oligonucleotide selected such as to correspond to a coherent sequence section of the TGF-β1 gene, placed at least one nucleotide upstream the 713-8C or 713-8delC, respectively, position of the gene (cf. Figs. 1 and 2a,b of the drawings), then digesting the PCR amplification product(s) thus produced with a restriction enzyme recognizing and cleaving the following nucleotide sequence:

```
CCA NNNNN TGG
GGT NNNNN ACC
```


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subjecting the digested product(s) to a restriction fragment length separation process and detecting whether a restriction fragment length corresponding to a cleavage of the PCR amplification product(s) is present or not.

Fig. 3

Description

[0001] The present invention relates to a method and kit for detecting a sequence variation in a gene. More particularly the invention relates to a method of detecting the presence or absence of a nucleotide sequense variation in the Transforming Growth Factor β1 gene (TGF-β1 gene). The invention also relates to particular oligonucleotide primers for use in PCR reactions performed e.g. in order to detect such nucleotide sequence variation. Also the invention concerns an oligonucleotide primer kit containing the particular primer(s).

BACKGROUND OF THE INVENTION:

[0002] Osteoporosis is a common disease with increasing rate of occurrence, characterized by reduced bone mass and increased fracture risk which affects up to 40% of women and 12% of men at some point during life. It would therefore be of importance to improve the capability of predicting the risk of osteoporosis earlier in life and thereby the possibility of instituting relevant prevention treatments.

[0003] In todays clinical practice, the evaluation of the risk of developing osteoporosis in an individual is based on measurements of bone strength and family history of osteoporosis.

[0004] Twin and family studies have shown that up to 75% of the age-specific variation in bone density is genetically determined. Osteoporosis is an example of a complex trait with a non-Mendelian way of inheritance. The phenotype of the individual is determined by a combination of genetic, environmental, dietary and hormonal factors. In order to characterize responsible genes, we and others have looked for the impact of polymorphisms and mutations in "candidate genes" on bone mass, bone turnover and prevalence of osteoporosis. Identification of such genes involved in the patogenesis of osteoporosis would greatly improve the estimate of risk.

POTENTIAL IMPACT OF TRANSFORMING GROWTH FACTOR β1 (TGF-β1):

[0005] TGF-β1 is released from bone matrix during bone resorption and subsequently activated by the low pH below the ruffled border of the resorbing osteoclasts. TGF-β1 has been implicated as a possible mediator of coupling between bone resorption and formation because:

1. TGF-β1 inhibits mature osteoclasts and proliferation of mononuclear osteoclast precursors in vitro,
2. the peptide inhibits fusion of mononuclear precursors into osteoclasts,
3. TGF-β1 has been found to stimulate proliferation or differentiation of preosteoblasts in vitro, and
4. bone matrix has the highest concentrations of TGF-β1 of all tissues.

[0006] The TGF-β1 gene consists of 7 exons, of which part of exon 5, exon 6 and exon 7 encode the active TGF-β1. TGF-β1 is produced and secreted as a propeptide of 390 amino acids by a variety of cell types, including osteoblasts. TGF-β1 is subsequently incorporated into bone matrix during matrix formation.

[0007] Derynck, R., Rhee, L., Chen, E.Y., and A. van Tilburg established in Nucl. Acids Res. 15 (7), pp. 3188-3189, (1987) the intron-exon structure and the complete base sequence of the human TGF-β1 precursor gene based on studies of two genomic DNA libraries, one derived from a normal fetal liver and one from the Calu-1 tumor cell line, cf. Fig. 1 of the attached drawings.

[0008] Based on polymerase chain reaction (PCR), polyamide gel electrophoresis (PGE) and semi-automated solid-phase dideoxynucleotide sequencing techniques Langdahl, B.L., Knudsen, J.Y. Jensen, H.K., Gregersen, N., and Eriksen, E.F. deseribe in Bone 20 (3), pp. 289-294, March 1997 the performance of a Single Stranded Conformation Polymorphism (SSCP) screening study of the TGF-β1 encoding gene derived from 161 osteoporotic women (i.e. having at least one low energy spinal fracture) and 131 normal women. The SSCP method reveals substitution(s) and/or deletion(s) of nucleic acid bases in the gene sequence examined.

[0009] Seven osteoporotic patients were found heterozygous for a cytosine to thymidine base substitution at position 76 in exon 5 (C^{788}-T)(corresponding to position 788 in the TGF-β1 cDNA), resulting in a threonine to isoleucine amino acid shift at position 263 in the TGF-β1 propeptide (Thr^{263}-Ile). Ten other osteoporotic patients had a one base deletion in the intron-4 sequence 8 bases prior to exon 5 (713-8delC, i.e. corresponding to position 713-8 in the TGF-β1 cDNA), that could influence splicing. Five normal women exhibited the C^{788}-T sequence variant and two the 713-8delC variant.

[0010] The prevalence of 713-8delC was significantly higher in the osteoporotic group (X^2=4.02, $p<0.05$). Osteoporotic patients with the 713-8delC variant had increased levels of bone alkaline phosphatase ($p<0.05$). If the osteoporotic patients with a Z-score below -1 in bone mineral density measurements of the lumber spine using dual-energy X-ray absorptiometri were examined separately, increased serum levels of bone alkaline phosphatase ($p<0.05$), increased urinary excretion of hydroxyproline ($p<0.05$) and reduced bone mass of the lumbar spine ($p<0.05$) were found in the patients with 713-8delC. No correlation to bone mass was demonstrated in the normal women, but 713-

8delC was associated with increased serum levels of bone alkaline phosphatase ($p<0.05$).

[0011] The sequence variation 713-8delC in the TGF-β1 gene is more frequent in patients with osteoporosis compared to normal controls. The 713-8delC variant seems to be associated with very low bone mass in osteoporotic women with low bone mass, and with increased bone turnover in both osteoporotic and normal women.

[0012] The above study thus also revealed that the base sequence established by Derynck et al. (supra) for the intron 4 domaine of the TGF-β1 gene was apparently based on an abnormal gene comprising a C-deletion at the 713-8 position, whereas the normal gene in fact comprises a C-nucleotide at the 713-8 position (i.e. the position referring to the TGF-β1 cDNA).

THE INVENTION:

[0013] Because the 713-8delC variation found in the above study could be a valuable genetic marker in the assessment of the risk for a given person to develop osteoporosis and the need for further research of the prevalence of this genetic variation and its occurence and impact in association with other osteoporotic risk factors there was a need for a quick and reliable method to detect this genetic 714-8delC variation.

[0014] The present invention provides a method which satisfies this need in all respects.

[0015] Thus, the present invention provides a method of detecting the presence or absence of the 713-8delC sequence variation in the human TGF-β1 gene, which method comprises provision of a sample comprising the TGF-β1 gene to be tested, or a relevant fragment thereof, subjecting the sample to a PCR amplification process using as upstream primer an oligonucleotide comprising a 15-100 nucleotides long section selected coherently from the following nucleotide sequence:

```
                *
3′ G TTT CGT ACC AAG TGA TGG CCG GCG
     GCT CCA CTG GAC CGG TGG TAA GTA
     CCG TAC TTG GCC GGA AAG GAC GAA
     GAG TAC CGG TGG GGC GAC CTC TCC
     CGG GTC GTA GAC 5′
```

such as to comprise the bold A* (the A marked with an asterisk) nucleotide, and as downstream primer a 15-100 nucleotides long oligenucleotide selected such as to correspond to a coherent sequence section of the TGF-β1 gene, placed at least one nucleotide upstream the 713-8C or 713-8delC, respectively, position of the gene (cf. Figs. 1 and 2a,b of the drawings), then digesting the PCR amplification product(s) thus produced with a restriction enzyme recognizing and cleaving the following nucleotide sequence:

```
CCA NNNNN TGG
GGT NNNNN ACC
```

subjecting the digested product(s) to a restriction fragment length separation process and detecting whether a restriction fragment length(s) corresponding to a cleavage of the PCR amplification product(s) is (are) present or not.

[0016] In case the digested PCR amplification product(s) is (are) completely cleaved this indicates that the analyzed TGF-β1 gene is normal, i.e. it has a C-nucleotide at the 713-8 position in both strands of the gene.

[0017] In case the digested PCR amplification product(s) is (are) only partly cleaved this indicates that the analyzed TGF-β1 gene is a heterozygote 713-8delC+/- variant, i.e. it has a C-nucleotide at the 713-8 position in one of the strands of the gene and a deletion of the C-nucleotide in that position in the other strand of the gene.

[0018] In case the digested PCR amplification product(s) is (are) uncleaved this indicates that the analyzed TGF-β1 gene is a homozygote 713-8delC+/+ variant, i.e. it has a deletion of the C-nucleotide at the 713-8 position in both strands of the gene.

[0019] By a "relevant fragment" of the TGF-β1 gene is meant a coherent nucleotide sequence section comprising the 713-8C or 713-8delC position and extending at least 15, preferably at least 20, nucleotides downstream from that position into the exon 5 domain and at least 15, preferably at least 25, more preferably at least 50, and most preferably at least 80, nucleotides upstream from that position. Preferably, a "relevant fragment" of the gene comprises a 100 to 500 nucleotides section of the gene. Preferably, the fragment comprises the whole exon 4 and intron 4 domains and at least a part of the consecutive exon 5 domain of the gene.

[0020] In the nucleotide sequence:

```
CCA NNNNN TGG
GGT NNNNN ACC
```

N means any one of the four nucleotiodes A, C, G, and T.

[0021] The cleaving arrows shown mean that the cutting enzyme used may cut the sequence at the posotions indicated as well as at any position between the two terminal groups shown, i.e. between:

```
CCA       TGG
     and
GGT       ACC
```

[0022] Preferably the upstream primer is 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

[0023] That the downstream primer is placed at least one nucleotide upstream the 713-8C or 713-8delC, respectively, position of the TGF-β1 gene means that the lowermost nucleotide at the 3' end af the primer is located at least one position upstream the 713-8C or 713-8delC, respectively, position of the gene. These positions refer to the TGF-β1 cDNA of the gene. However, the lowermost nucleotide at the 3' end of the primer is preferably at least located 25 nucleotide positions, more preferably at least 50 nucleotide positions, yet more preferably at least 100 nucleotide positions, and most preferably at least 150 nucleotide positions upstream the 713-8 position of the gene. Preferably the downstream primer is 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

[0024] The upstream and downstream primers are preferably selected such that the amplification products produced by the PCR method of the invention are at least 80 nucleotides long, preferably from 100 to 500 nucleotides long.

[0025] In addition to the particular primers used in the PCR amplification process also the usual other compounds and components used in such a process will be present such as the four mononucleotides dATP, dCTP, dGTP and dTTP, necessary salts like $MgCl_2$, buffer(s), and a polymerase enzyme like Taq polymerase, E-coli DNA-polymerase I, the Klenow fragmant of E-coli, T_4-DNA polymerase and other available suitable polymerases.

[0026] Preferred embodiments of the method are set forth in the attached claims 2-9.

[0027] The invention also concerns an oligonucleotide primer comprising a 15-100 nucleotides long section selected coherently from the nucleotide sequence:

```
            *
3' G TTT CGT ACC AAG TGA TGG CCG GCG
     GCT CCA CTG GAC CGG TGG TAA GTA
     CCG TAC TTG GCC GGA AAG GAC GAA
     GAG TAC CGG TGG GGC GAC CTC TCC
     CGG GTC GTA GAC  5'
```

such as to comprise the bold **A*** (the A marked with the asterisk).

[0028] Preferably, this oligonucleotide primer is 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

[0029] A particular preferred primer is:

```
           *
3' TT CGT ACC AAG TGA TGG CCG GCG 5'
```

[0030] Furthermore, the invention concerns a primer kit comprising an oligonucleotide primer as defined above (cf. claims 10-13) and another oligonucleotide primer comprising a 15-100 nucleotides long sequence corresponding to a coherent sequence section of the TGF-β1 gene placed at least one nucleotide upstream the 713-8C or 713-8delC, respectively, position of the TGF-β1 gene (cf. Figs. 1 and 2a,b of the drawings). The second oligonucleotide primer of the kit is preferably 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

[0031] A preferred primer kit comprises a primer which is:

```
           *
3' TT CGT ACC AAG TGA TGG CCG GCG 5'
```

and another primer which is

5' ATT GAG GGC TTT CGC CTT AGC GC 3'

[0032] The above primers may have other mismatching nucleotides incorporated in their sequence than the above indicated obligatory one (the bold **A** marked with the asterisk) provided such further mismatching nucleotide(s) do not impair or prevent their function as primers essentially and do not result in PCR products which when digested by the restriction enzyme lead to misleading restriction fragment lengths. Also on the same conditions the primers may be attached to non-complementary nucleotide fragments or particular markers or marker groups at their 5' ends.

[0033] Finally, the invention also concerns any DNA sequence comprising the sequence section:

```
           *
3' TT CGT ACC AAG TGA TGG CCG GCG 5'
```

as well as any primer pair comprising the DNA sequence. Preferably the DNA sequence comprises no more than 100 nucleotides, more preferably no more than 50 nucleotides, yet more preferably no more than 30 nucleotides, and at the

3' end it has preferably no more than 7 nucleotides exceeding beyond the bold A* (the A marked with an asterisk).

DETAILED EXPLANATION OF THE THEORY ON WHICH THE INVENTION IS BASED:

[0034] Neither the 713-8C position of the TGF-β1 gene nor the 713-8delC variation thereof do themselves constitute natural restriction sites for any known endonuclease restriction enzyme. Therefore the particular idea was to investigate whether it would be possible by PCR technique to produce artificial nucleotiode amplification products which would be distinctive and discernible for one or more known endonuclease restriction enzymes depending on the fact whether the amplification product was derived from the normal 713-8C TGF-β1 gene or from the 713-8del C variation thereof.

[0035] The investigation instituted turned out to be successfull and the solution found will be explained in more details in the following on the basis of two particular primers and the particular restriction enzyme Van 91I.

[0036] The normal sequence of the intron 4 leading up to the exon 5 domain in the TGF-β1 gene is:

5' cccacac<u>c</u>aaagcagGG TTC ACT 3'

small letters: intron sequence, and capital letters: exon sequence, the underlined <u>c</u> represents the 713-8C position.

[0037] The sequence variant 713-8delC has a deletion at position 8 before the exon 5:

5' cccacac_aaagcagGG TTC ACT 3'

[0038] To detect the deletion the following primers are constructed:

Downstream: 5' ATT GAG GGC TTT CGC CTT AGC GC 3' (located in exon 4)

Upstream: 5' GCG GCC GGT AGT GAA CC**A** TGC TT 3' (located partly in intron 4 and partly in exon 5)

[0039] The Upstream primer has a mismatch (bold type **A**) when attached to the TGF-β1 gene at the appropriate portion:

```
cccacac_caaagcagGG TTC ACT ACC GGC CGC CGA GGT...
        3' TTCGTACC AAG TGA TGG CCG GCG 5'
```

[0040] The PCR-products are thus,
in the normal sequence:

...CC CAC ACC AAA GCA **T**GG TTC ACT ACC GGC CGC

in the variant sequence:

...CC CAC AC_ AAA GCA **T**GG TTC ACT ACC GGC CGC

[0041] (Bold type T introduced due to the presence of the mismatching bold type **A** in the upstream primer). And the final PCR amplification products, which will be double-stranded, are:

Normal:

[0042]

...CC CAC ACC AAA GCA **T**GG TTC ACT ACC GGC CGC
...GG GTG TGG TTT CGT **A**CC AAG TGA TGG CCG GCG

Variant:

[0043]

...CC CAC AC AAA GCA TGG TTC ACT ACC GGC CGC
...GG GTG TG TTT CGT **A**CC AAG TGA TGG CCG GCG

[0044] The restriction enzyme Van 91I recognizes and cuts the following nucleotide sequence:

```
CCA NNNNN TGG
GGT NNNNN ACC
```

where N can be any of the four nucleotides A, C, G, and T. Hence, the normal PCR product will be cleaved by the enzyme, the variant PCR product not:

Normal:

clevage

```
...CC CAC ACC AAA GCA TGG TTC ACT ACC GGC CGC
...GG GTG TGG TTT CGT ACC AAG TGA TGG CCG GCG
```

Variant:

no clevage

```
...CC CAC AC AAA GCA TGG TTC ACT ACC GGC CGC
...GG GTG TG TTT CGT ACC AAG TGA TGG CCG GCG
```

[0047] The invention will now be further explained with reference to the following example and the attached drawings in which:

- Fig. 1 shows the downstream strand nucleotide sequence of the toal TGF-β1 gene as established by Derynck et al. (supra),
- Fig. 2a shows the downstream strand nucleotide sequence of the whole exon 4, intron 4 and exon 5 of the normal TGF-β1 gene as established by the present inventors, i.e. containing the normal c-nucleotide at the 713-8 position, and parts of the introns 3 and 5,
- Fig. 2b shows the downstream strand nucleotide sequence of the whole exon 4, intron 4 and exon 5 of the 713-8delC variant of the TGF-β1 gene as established by the present inventors, i.e. having a deletion of the c-nucleotide at the 713-8 position, and parts of the introns 3 and 5,
- Fig. 3 is a photograph of an electrophoretic separation pattern in a 3% agarose gel of non-digested and digested PCR products produced by the method of the invention and showing seven separation lanes, the first one of which from the left being a marker lane, and

Fig. 4 is a sketch of an electrophoretic separation pattern of another run in 3% agarose gel of non-digested and digested PCR products produced by the method of the invention and showing with greater clarity seven separation lanes, the first one of which from the left being a marker lane.

EXAMPLE

[0048] Whole blood samples were collected from osteoporotic and normal women. DNA was isolated from whole blood leukocytes using a QIAamp Blood Kit® from QIAGEN®. The procedure used in obtaining purified DNA from the samples is described in the QIAamp Blood KIT and QIAamp Tissue Kit Handbook (December 1996), QIAGEN Inc., 28159 Avenue Stanford, Santa Clarita, CA 91355-1106, USA.

[0049] Polymerase chain reactions (PCR) were performed in a final volume of 50 μl using:

5 μl 10xPCR buffer (Perkin Elmer)
3 μl $MgCl_2$ 15 mM (Perkin Elmer)
0,1 μmol of each NTP (Perkin Elmer)
1,25 U Taq Polymerase (Perkin Elmer)
25 pmol upstream primer[a)] and 25 pmol dowmstream primer[b)]
2 μl DNA (100 ng/μl)
31 μl H_2O

[0050] The final PCR mixture was overlaid with 30 μl mineral oil. PCR was conducted in an automated Perkin Elmer/Cetus Thermal Cycler model 480 at the following conditions:

Initial denaturation:	10 min. at 95° C.
35 Cycles:	Denaturation at 95° C for 1 min. Annealing at 61° C for 1 min. Primer extension at 74° C for 1 min.
Final primer extension:	8 min at 74° C.

DIGESTION:

[0051] Samples of the PCR products produced were digested at 37° C for 2 hours using:

10 μl of the PCR product
0,5 μl Van 911 (5U/μl) (Boehringer Mannheim)
2 μl buffer B (Boehringer Mannheim)
8 μl H_2O

ELECTROPHORESIS:

[0052] 10 μl of non-digested and digested, respectively, PCR amplificatin products were placed in slots of a 3% agarose gel. As was 10 μl of a size marker solution from Boehringer-Mannheim designated "DNA Molecular Weight Marker V" and containing 22 DNA fragments of from 8 to 587 base-pairs in length spaced in groups of five different fragment base-pair lengths. The running buffer was a Tris-borate EDTA buffer (Tris-borate: 0.89 M, EDTA: 0.02 M), pH = 8.0, and electrohoretic separation was performed at 100V for 2 hours.

a) upstream primer was:
5' GCG GCC GGT AGT GAA CC**A** TGC TT 3'
b) downstream primer was:
5' ATT GAG GGC TTT CGC CTT AGC GC 3'

[0053] The electrophoretic separation pattern was visualized by staining the gel with ethidium bromide and visualization effected under UV-light.

RESULTS AND DISCUSSION

[0054] Fig. 3, which is a photograph of an electrophoretic separation pattern in a 3% agarose gel of non-digested and digested PCR amplification products produced by the method of the invention, shows seven separation lanes, which being from the left to the right as follows:

lane 1:	size marker sample showing bands of DNA fragments of from 64 (uppermost) to 587 (lowermost) base-pairs in length, the group of bands in line horizontally with the bands in the other lanes represents DNA fragments of 267 (lowermost), 234, 213, 192, and 184 (uppermost, bands 192 and 184 being seen as one single broad band) base-pairs, supplied from Boehringer-Mannheim under the designation "DNA Molecular Weight Marker V"
lane 3	: non-digested (i.e. uncleaved) normal TGF-β1 gene
lane 4:	digested (i.e. cleaved) normal TGF-β1 gene
lane 6:	non-digested heterozygote 713-8delC+/- TGF-β1 gene
lane 7:	digested (partly cleaved) heterozygote 713-8delC+/- TGF-β1 gene
lane 9:	non-digested homozygote 713-8delC+/+ TGF-β1 gene
lane 10:	digested homozygote 713-8delC+/+ TGF-β1 gene
lanes 2, 5, 8, and 11-13	being empty.

[0055] Lanes 3, 6, and 9 show that irrespective of whether the TGF-β1 gene is normal, a heterozygote or homozygote 713-8delC variant, the non-digested PCR amplification products produced by using a primer set according to the present invention will only produce one single band at substantially the same distance from the origin after subjection to electrophoretic separation; the band in lane 3, however, being retarded a minor distance for unaccountable experimental reasons.

[0056] Lane 4 shows that digested PCR amplification products produced from the normal TGF-β1 using a primer set according to the present invention will be completely cleaved (i.e. the PCR products of both strands of the gene cleaved) by the restriction enzyme used in the method of the invention leaving only one single, but displaced band (as compared to the same non-digested, uncleaved normal gene PCR products) after electrophoretic separation.

[0057] Lane 7 shows that digested PCR amplification products produced from the heterozygote 713-8delC+/- TGF-β1 gene using a primer set according to the present invention will be only partly cleaved (i.e. the PCR products of one strand of the gene cleaved, the PCR products of the other strand of the gene uncleaved) by the restriction enzyme used in the method of the invention leaving two distinct bands, one of which being displaced, the other being located at substantially the same position (as compared to the same non-digested, uncleaved gene PCR products) after electrophoretic separation.

[0058] Lane 10 shows that digested PCR amplification products produced from the homozygote 713-8delC+/+ TGF-β1 gene using a primer set according to the present invention will not be cleaved by the restriction enzyme used in the method of the invention leaving only one distinct band being located at substantially the same position (as compared to the same non-digested (uncleaved) gene PCR products) after electrophoretic separation.

[0059] Fig. 4, which is a sketch of another electrophoretic separation pattern run in similar manner in a 3% agarose gel as the electrophoretic separation patern shown in Fig. 3, of non-digested and digested PCR amplification products produced by the method of the invention, shows with greater clarity the same features as explained above for Fig. 3, except that another size marker sample is used and that the empty lanes 11-13 are absent from the sketch. Thus the following lanes are shown:

lane 1:	size marker sample comprising DNA fragments mutually spaced by about 25 base-pairs
lane 3:	non-digested (i.e. uncleaved) normal TGF-β1 gene
lane 4:	digested (i.e. cleaved) normal TGF-β1 gene
lane 6:	non-digested heterozygote 713-8delC+/- TGF-β1 gene
lane 7:	digested (partly cleaved) heterozygote 713-8delC+/- TGF-β1 gene
lane 9:	non-digested homozygote 713-8delC+/+ TGF-β1 gene
lane 10:	digested homozygote 713-8delC+/+ TGF-β1 gene
lanes 2, 5 and 8	being empty

CONCLUDING REMARKS

[0060] Electrophoresis of digested PCR amplification products should always be run together with a non-digested (i.e. uncleaved) sample as a negative control.

[0061] After isolation of the DNA to be tested the PCR can be performed within 3-4 hours, digestion takes about 2 hours, and electrophoresis takes about another 2 hours. Thus, the total assay time takes about 7-8 hours. The number of samples run simultaneously depends on the PCR Thermocycler, but with one having a capacity of 96 samples per run it will be possible to genotype 95 + 1 negative control in one day.

[0062] The PCR runs very stably with the primers disclosed herein and the digesting assay results in 100 percent reproducible results.

[0063] Thus, having described the invention in both general and detailed terms the scope of the invention for which protection is applied, is defined in the attached claims. However, it should be understood that other features and modifications than those particularly described in this specification and claims, which are evident for the man skilled in the art and which will be advantageous in connection with the performance of the present invention, are also considered to be within the scope of the present invention.

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(1) GENERAL INFORMATION:

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          (F) POSTAL CODE (ZIP): 8230

    (ii) TITLE OF INVENTION: Method and kit for detecting a sequence
           variation in a gene

   (iii) NUMBER OF SEQUENCES: 2

    (iv) COMPUTER READABLE FORM:
          (A) MEDIUM TYPE: Floppy disk
          (B) COMPUTER: IBM PC compatible
          (C) OPERATING SYSTEM: PC-DOS/MS-DOS
          (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

     (v) CURRENT APPLICATION DATA:
          APPLICATION NUMBER: EP 98106224.3

(2) INFORMATION FOR SEQ ID NO: 1:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 109 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO


     (x) PUBLICATION INFORMATION:
          (A) AUTHORS: Langdahl, B.L.
                       Knudsen, J.Y.
                       Jensen, H.K.
                       Gregersen, N.
                       Eriksen, E.F.
          (C) JOURNAL: Bone
          (D) VOLUME: 20
          (F) PAGES: 289-294
          (G) DATE: March-1997
     (x) PUBLICATION INFORMATION:
          (A) AUTHORS: Derynck, R.
                       Rhee, L.
                       Chen, E.Y.
                       van Tilburg, A.
          (C) JOURNAL: Nucleic Acids Res.
          (D) VOLUME: 15
          (F) PAGES: 3188-3189
          (G) DATE: 1987

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CAGATGCTGG GCCCTCTCCA GCGGGGTGGC CATGAGAAGC AGGAAAGGCC GGTTCATGCC
60
```

ATGAATGGTG GCCAGGTCAC CTCGGCGGCC GGTAGTGAAC CATGCTTTG
109

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Langdahl, B.L.
Knudsen, J.Y.
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Gregersen, N.
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(A) AUTHORS: Derynck, R.
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GCGGCCGGTA GTGAACCATG CTT
23

Claims

1. A method of detecting the presence or absence of the 713-8delC sequence variation in the human TGF-β1 gene, which method comprises provision of a sample comprising the TGF-β1 gene to be tested, or a relevant fragment thereof, subjecting the sample to a PCR amplification process using as upstream primer an oligonucleotide comprising a 15-100 nucleotides long section selected coherently from the following nucleotide sequence:

*

3′ G TTT CGT **A**CC AAG TGA TGG CCG GCG
GCT CCA CTG GAC CGG TGG TAA GTA
CCG TAC TTG GCC GGA AAG GAC GAA
GAG TAC CGG TGG GGC GAC CTC TCC
CGG GTC GTA GAC 5′

such as to comprise the bold **A*** (marked with the asterisk) nucleotide, and as downstream primer a 15-100 nucleotides long oligonucleotide selected such as to correspond to a coherent sequence section of the TGF-β1 gene, placed at least one nucleotide upstream the 713-8C or 713-8delC, respectively, position of the gene (cf. Figs. 1 and 2a,b of the drawings), then digesting the PCR amplification product(s) thus produced with a restriction enzyme recognizing and cleaving the following nucleotide sequence:

CCA NNNNN TGG
GGT NNNNN ACC

subjecting the digested product(s) to a restriction fragment length separation process and detecting whether a restriction fragment length(s) corresponding to a cleavage of the PCR amplification product(s) is (are) present or not.

2. A method according to claim 1, wherein the upstream primer is 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

3. A method according to claims 1 or 2, wherein the downstream primer is 15-50, more prefereblу 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

4. A method according to claim 1, wherein the upstream primer is:

*

3′ TT CGT **A**CC AAG TGA TGG CCG GCG 5′

and the downstream primer is:

5' ATT GAG GGC TTT CGC CTT AGC GC 3'

5. A method according to any of the preceding claims, wherein the upstream and downstream primers are selected such that the amplification products produced by the PCR method of the invention are at least 80 nucleotides long, preferably from 100 to 500 nucleotides long.

6. A method according to any of the preceding claims, wherein the upstream and/or the downstream primer have other mismatching nucleotides incorporated in their sequence than the above in claim 1 indicated obligatory one (the bold **A** marked with the asterisk*) provided such further mismatching nucleotide(s) does (do) not impair or prevent their function as primers substantially and does (do) not result in PCR amplification products which when digested by the restriction enzyme lead to misleading restriction fragment lengths, and optionally, on the same conditions the primers may be attached to non-complementary nucleotide fragments or particular markers or marker

groups at their 5' ends.

7. A method according to any of the preceding claims, wherein the restriction enzyme is Van 91I, PflMI or AccB7I.

8. A method according to any of the preceding claims wherein the PCR amplification is effected with Taq polymerase and the PCR conditions are approximately:

Initially:	10 min at 95°C
Cycles:	(1 min at 95°C + 1 min at 61°C + 1 min at 74°C) x 35
Finally:	8 min at 74°C.

9. A method according to any of the preceding claims, wherein the restriction fragment length separation process is an electrophoretic separation process, preferably performed in agarose gel.

10. An oligonucleotide primer comprising a 15-100 nucleotides long section selected coherently from the following nucleotide sequence:

```
                *
3' G TTT CGT ACC AAG TGA TGG CCG GCG
     GCT CCA CTG GAC CGG TGG TAA GTA
     CCG TAC TTG GCC GGA AAG GAC GAA
     GAG TAC CGG TGG GGC GAC CTC TCC
     CGG GTC GTA GAC  5'
```

such as to comprise the bold **A*** (the A marked with the asterisk) nucleotide.

11. An oligonucleotide primer according to claim 10, said primer being 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

12. An oligonucleotide primer according to claim 10, said primer being:

```
             *
3' TT CGT ACC AAG TGA TGG CCG GCG 5'
```

13. An oligonucleotide primer according to any of claims 10-12, wherein said primer has other mismatching nucleotides incorporated in its sequence than the above in claim 10 indicated obligatory one (the bold **A** marked with the asterisk*) provided such further mismatching nucleotide(s) does (do) not impair or prevent its function as primer substantially and does (do) not result in PCR amplification products which when digested by a restriction enzyme lead to misleading restriction fragment lengths, and optionally, on the same conditions the said primer may be attached to non-complementary nucleotide fragments or particular markers or marker groups at its 5' end.

14. A primer kit comprising an oligonucleotide first primer according to any of claims 10-13 and another oligonucleotide second primer comprising a 15-100 nucleotides long sequense corresponding to a coherent sequence section of the TGF-β1 gene selected at least one nucleotide upstream the 713-8C or 713-8delC, respectively, position of the TGF-β1 gene (cf. Figs. 1 and 2a,b of the drawings).

15. A primer kit according to claim 14, wherein the first primer is 15-50, more preferably 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long, and the second primer is 15-50, more preferebly 15-40, yet more preferably 15-30, and most preferably 20-30, nucleotides long.

16. A primer kit according to claim 14, wherein the first primer is:

*
3' TT CGT **A**CC AAG TGA TGG CCG GCG 5'

and the second primer is:

5' ATT GAG GGC TTT CGC CTT AGC GC 3'.

17. A primer kit according to any of claims 14-16, wherein the first and/or the second primer have other mismatching nucleotides incorporated in their sequence than the above in claim 10 indicated obligatory one (the bold **A** marked with the asterisk*) provided such further mismatching nucleotide(s) does (do) not impair or prevent their function as primers substantially and does (do) not result in PCR amplification products which when digested by a restriction enzyme lead to misleading restriction fragment lengths, and optionally, on the same conditions the primers may be attached to non-complementary nucleotide fragments or particular markers or marker groups at their 5' ends.

Fig. 1

```
1    CTGCAGGCTGGCCCCGGCTCCATTTCCAGGTGTGGTCCCAGGACAGCTTTGGCCGCTGCCAGCTTGCAGACTATGGATTTTGCCATGTGCCCAGTAGCCC
101  GGGCACCCACCAGCTGGCCTGCCCCACGTGGCGGCCCCTGGGCAGTTGGCGAGAACAGTTGGCACGGGCTTTCGTGGGTGGTGGCCCCGCAGCTGCTGCAT
201  GGGGACACCATCTACAGTGGGGCCGACCGCTATCGCCTGCACACAGCTGCTGGTGGCACCGTGCACCTGGAGATCGGCCTGCTGCTCCGCAACTTCGACC
301  GCTACGGCGTGGAGTGCTGAGGG..CTCTGCCTCCAACGTCACCACCATCCACACCCCGGACACCCAGTGATGGGGGAGGATGGCACAGTGGTCAAGAGCA
401  CAGACTCTAGAGACTGTCAGAGCTGACCCCAGCTAAGGCATGGCACCGCTTCTGTCCTTTCTAGGACCTCGGGGTCCCTCTGGGCCCAGTTTCCCTATCT
501  GTAAATTGGGGACAGTAAATGTATGGGGTCGCAGGGTGTTGAGTGACAGGAGGCTGCTTAGCCACATGGGAGGTGCTCAGTAAAGGAGAGCAATTCTTAC
601  AGGTGTCTGCCTCCTGACCCTTCCATCCCTCAGGTGTCCTGTTGCCCCCTCCTCCCACTGACACCCTCCGGAGGCCCCCATGTTGACAGACCCTCCTTCT
701  CCTACCTTGTTTCCCAGCCTGACTCTCCTTCCGTTCTGGGTCCCCCTCCTCTGGTCGGCTCCCCTGTGTCTCATCCCCCGGATTAAGCCTTCTCCGCCTG
801  GTCCTCTTTCTCTGGTGACCCACACCGCCCGCAAAGCCACAGCGCATCTGGATCACCCGCTTTGGTGGCGCTTGGCCGCCAGGAGGCAGCACCCTGTTTG
901  CGGGGCGGAGCCGGGGAGCCCCGCCCCCTTTCCCCCAGGGCTGAAGGGACCCCCCTCGGAGCCCGCCCACGCGAGATGAGGACGGTGGCCCAGCTCCCCCA
1001 TGCCCTCCCCCTGGGGGCCGCCCCCGCTCCCGCCCCGTGCGCTTCCTGGGTGGGGCCGGGGGCGGCTTCAAAACCCCCTGCCGACCCAGCCGGTCCCCGC
1101 CGCCGCCGCCCTTCGCGCCCTGGGCCATCTCCCTCCCACCTCCCTCCGCGGAGCAGCCCAGACAGCGAGGGCCCCGGCCGGGGGCAGGGGGGACGCCCCGT
1201 CCGGGGCACCCCCCCGGCTCTGAGCCGCCCGCGGGGCCGGCCTCGGCCCGGAGCGGAGGAAGGAGTCGCCGAGGAGCAGCCTGAGCCCCAGAGTCTGAG
1301 ACGAGCCGCCGCCGCCCCCGCCACTGCGGGGAGGAGGGGGCAGGAGGAGCGGGAGGAGGGACGAGCTGGTCGGGAGAAAGGAAAAAAACTTTTGAGACTT
1401 TTCCGTTGCCGCTGGGAGCCGGAGGCGCGGGGACCTCTTGGCGCGACGCTGCCCCGCGAGGAGGCAGGACTTGGGGACCCCAGACCGCCTCCCTTTGCCG
1501 CCGGGGACGCTTGCTCCCTCCCTGCCCCCTACACGGCGTCCCTCAGGCGCCCCCATTCCGGACCAGCCCTCGGGAGTCGCCGACCCGGCCTCCCGCAAAG
1601 ACTTTTCCCCAGACCTCGGGCGCACCCCCTGCACGCCGCCTTCATCCCCGGCCTGTCTCCTGAGCCCCCGCGCATCCTAGACCCTTTCTCCTCCAGGAGA
1701 CGGATCTCTCTCCGACCTGCCACAGATCCCCTATTCAAGACCACCCACCTTCTGGTACCAGATCGCGCCCATCTAGGTTATTTCCGTGGGATACTGAGAC
1801 ACCCCCGGTCCAAGCCTCCCCTCCACCACTGCGCCCTTCTCCCTGAGGACCTCAGCTTTCCCTCGAGGCCCTCCTACCTTTTGCCGGGAGACCCCCAGCC

                                                                                           1
                                                                                           Met Pro Pro Ser Gly
1901 CCTGCAGGGGCGGGGCCTCCCCACCACACCAGCCCTGTTCGCGCTCTCGGCAGTGCCGGGGGCGCCGCCTCCCCC          ATG CCG CCC TCC GGG

             10                                      20                                      30
     Leu Arg Leu Leu Leu Leu Leu Leu Pro Leu Leu Trp Leu Leu Val Leu Thr Pro Gly Arg Pro Ala Ala Gly Leu
1992 CTG CGG CTG CTG CTG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG CCT GGC CGG CCG GCC GCG GGA CTA

                                             40                                      50
     Ser Thr Cys Lys Thr Ile Asp Met Glu Leu Val Lys Arg Lys Arg Ile Glu Ala Ile Arg Gly Gln Ile Leu Ser
2067 TCC ACC TGC AAG ACT ATC GAC ATG GAG CTG GTG AAG CGG AAG CGC ATC GAG GCC ATC CGC GGC CAG ATC CTG TCC

             60                                      70                                      80
     Lys Leu Arg Leu Ala Ser Pro Pro Ser Gln Gly Glu Val Pro Pro Gly Pro Leu Pro Glu Ala Val Leu Ala Leu
2142 AAG CTG CGG CTC GCC AGC CCC CCG AGC CAG GGG GAG GTG CCG CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG

                                             90                                      100
     Tyr Asn Ser Thr Arg Asp Arg Val Ala Gly Glu Ser Ala Glu Pro Glu Pro Glu Pro Glu Ala Asp Tyr Tyr Ala
2217 TAC AAC AGC ACC CGC GAC CGG GTG GCC GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC TAC TAC GCC

             110
     Lys Glu Val Thr Arg Val Leu Met Val Glu Thr His Asn G
2292 AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC Ggtgagctcggaggggcaggggagccgggaggggggcccccagggg
```

Fig. 1 cont.

120 130
Iu Ile Tyr Asp Lys Phe Lys Gln Ser Thr His Ser Ile Tyr
cgcc............gatcgcctcccttcatttctccctgctagAA ATC TAT GAC AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT

140 150
Met Phe Phe Asn Thr Ser Glu Leu Arg Glu Ala Val Pro Glu Pro Val Leu Leu Ser Arg Ala Glu Leu Arg Leu
ATG TTC TTC AAC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG CGT CTG

160 170
Leu Arg Leu Lys Leu Lys Val Glu Gln His Val Glu Leu Tyr Gln
CTG AGG CTC AAG TTA AAA GTG GAG CAG CAC GTG GAG CTG TAC CAG gtgaggacatgagccagaaggaaggtcagggcatgggct

Lys Tyr Ser Asn Asn Ser Trp
ggagagggtga..........tcgtgacaccatctacccactgtctctctcctgccttcatcatcctctag AAA TAC AGC AAC AAT TCC TGG

180 190 200
Arg Tyr Leu Ser Asn Arg Leu Leu Ala Pro Ser Asp Ser Pro Glu Trp Leu Ser Phe Asp Val Thr Gly Val Val
CGA TAC CTC AGC AAC CGG CTG CTG GCA CCC AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC GGA GTT GTG

210
Arg Gln Trp Leu Ser Arg Gly G
CGG CAG TGG TTG AGC CGT GGA Ggtgaggattacttgtgtgtcccacccctgttttctccctggggtccaccc...........gctgggtga

220
ly Glu Ile Glu Gly Phe Arg Leu Ser Ala His Cys Ser Cys Asp
gctgcactctcagactggcttccctctcgccactcctacagGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC TGC TCC TGT GAC

230
Ser Arg Asp Asn Thr Leu Gln Val Asp Ile Asn G
AGC AGG GAT AAC ACA CTG CAA GTG GAC ATC AAC Ggtgaggcctgcttccccggccatgcccagttgtgacgtgtgtgcgtgtgtgtgt

240
ly Phe Thr Thr
tccccatctgccccacgccccacttatctatccctctgagagtgtgtgtgtatgtcccctatcccctgactccccacacaagcagGG TTC ACT ACC

250 260
Gly Arg Arg Gly Asp Leu Ala Thr Ile His Gly Met Asn Arg Pro Phe Leu Leu Leu Met Ala Thr Pro Leu Glu
GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG

270 280
Arg Ala Gln His Leu Gln Ser Ser Arg His Arg Arg Ala Leu Asp Thr Asn Tyr Cys Phe Se
AGG GCC CAG CAT CTG CAA AGC TCC CGG CAC CGC CGA GCC CTG GAC ACC AAC TAT TGC TTC AGgtgagccttgtagcctg

r Ser
gatggaggccttccaggctggggcatgactgc...........tctatggtggtagcccctccctgcccctgatgcgtctctcctgcctgcagC TCC

290 300 310
Thr Glu Lys Asn Cys Cys Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys Asp Leu Gly Trp Lys Trp Ile His Glu
ACG GAG AAG AAC TGC TGC GTG CGG CAG CTG TAC ATT GAC TTC CGC AAG GAC CTC GGC TGG AAG TGG ATC CAC GAG

320 330
Pro Lys Gly Tyr His Ala Asn Phe Cys Leu Gly Pro Cys Pro Tyr Ile Trp Ser Leu Asp Thr Gln Tyr Ser Lys
CCC AAG GGC TAC CAT GCC AAC TTC TGC CTC GGG CCC TGC CCC TAC ATT TGG AGC CTG GAC ACG CAG TAC AGC AAG

gtacgtctggccaccgggctacgagatgcgcttgggggggagccaggacgg..........ttttcctccctcccacgagccctgagccctgaccccgccc

340 350 360
Val Leu Ala Leu Tyr Asn Gln His Asn Pro Gly Ala Ser Ala Ala Pro Cys Cys Val Pro Gln Ala
gccgccccgcag GTC CTG GCC CTG TAC AAC CAG CAT AAC CCG GGC GCC TCG GCG GCG CCG TGC TGC GTG CCG CAG GCG

370 380
Leu Glu Pro Leu Pro Ile Val Tyr Tyr Val Gly Arg Lys Pro Lys Val Glu Gln Leu Ser Asn Met Ile Val Arg
CTG GAG CCG CTG CCC ATC GTG TAC TAC GTG GGC CGC AAG CCC AAG GTG GAG CAG CTG TCC AAC ATG ATC GTG CGC

390
Ser Cys Lys Cys Ser
TCC TGC AAG TGC AGC TGA GGTCCCGCCCCGCCCCGCCCCGCCCCGGCAGGCCCGGCCCCACCCCGCCCCGCCCCCGCTGCCTTGCCCATGGGG

GCTGTATTTAAGGACACCCGTGCCCCAAGCCCACCTGGGGCCCCATTAAAGATGGAGAGAGGACTGCGGATCTCTGTGTCATTGGGCGCCTGCCTGGGGT

CTCCATCCCTGACGTTCCCCCACTCCCACTCCCTCTCTCTCCCTCTCTGCCTCCTCCTGCCTGTCTGCACTATTCCTTTGCCCGGCATCAAGGCACAGGG

GACCAGTGGGGAACACTACTGTAGTTAGATCTATTTATTGAGCACCTTGGGCACTGTTGAAGTGCCTTACATTAATGAACTCATTCAGTCACCATAGCAA

CACTCTGAGATGCAGGGACTCTGATAACACCCATTTTAAAGGTGAGGAAACAAGCCCAGAGAGGTTAAGGGAGGAGTTCCTGCCCACCAGGAACCTGCTT

TAGTGGGGGATAGTGAAGAAGACAATAAAAGATAGTAGTTCAGGCCAGGCGGGGTGGCTCACGCCTGTAATCCTAGCACTTTTGGGAGGCAGAGATGGGA

GGATTACTTGAATCCAGGCATTTGAGACCAGCCTGGGTAACATAGTGAGACCCTATCTCTACAAAACACTTTTAAAAAATGTACACCTGTGGTCCCAGCT

ACTCTGGAGGCTAAGGTGGGAGGATCACTTGATCCTGGGAGGTCAAGGCTGCAG

Fig. 2a

```
                                                        5'.....gctgggtgagctg
                                          intron 3-| |-exon 4
cactctcagactggcttccctctcgccactcctacagGG GAA ATT GAG GGC TTT
CGC CTT AGC GCC CAC TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG
                 exon 4-| |-intron 4
CAA GTG GAC ATC AAC Ggtgaggcctgcttccccggccatgcccagttgtgac
gtgtgtgcgtgtgtgtgttcccatctgccccacgccccacttatctatccctctgaga
                                  position 713-8-| intron 4-| |-exon 5
gtgtgtgtgtatgtcccctatcccctgactcccacaccaaagcagGG TTC ACT ACC
GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT
TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GCC CAG CAT CTG
CAA AGC TCC CGG CAC CGC CGA GCC CTG GAC ACC AAC TAT TGC TTC
exon 5-| |-intron 5
AGgtgagccttgtagcctggatggaggccttccaggctgggggcatgactgc.....3'
```

Fig. 2b

```
                                                        5'.....gctgggtgagctg
                                          intron 3-| |-exon 4
cactctcagactggcttccctctcgccactcctacagGG GAA ATT GAG GGC TTT
CGC CTT AGC GCC CAC TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG
                 exon 4-| |-intron 4
CAA GTG GAC ATC AAC Ggtgaggcctgcttccccggccatgcccagttgtgac
gtgtgtgcgtgtgtgtgttcccatctgccccacgccccacttatctatccctctgaga
                                  position 713-8-| intron 4-| |-exon 5
gtgtgtgtgtatgtcccctatcccctgactcccacac_aaagcagGG TTC ACT ACC
GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT
TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GCC CAG CAT CTG
CAA AGC TCC CGG CAC CGC CGA GCC CTG GAC ACC AAC TAT TGC TTC
exon 5-| |-intron 5
AGgtgagccttgtagcctggatggaggccttccaggctgggggcatgactgc.....3'
```

Fig. 3

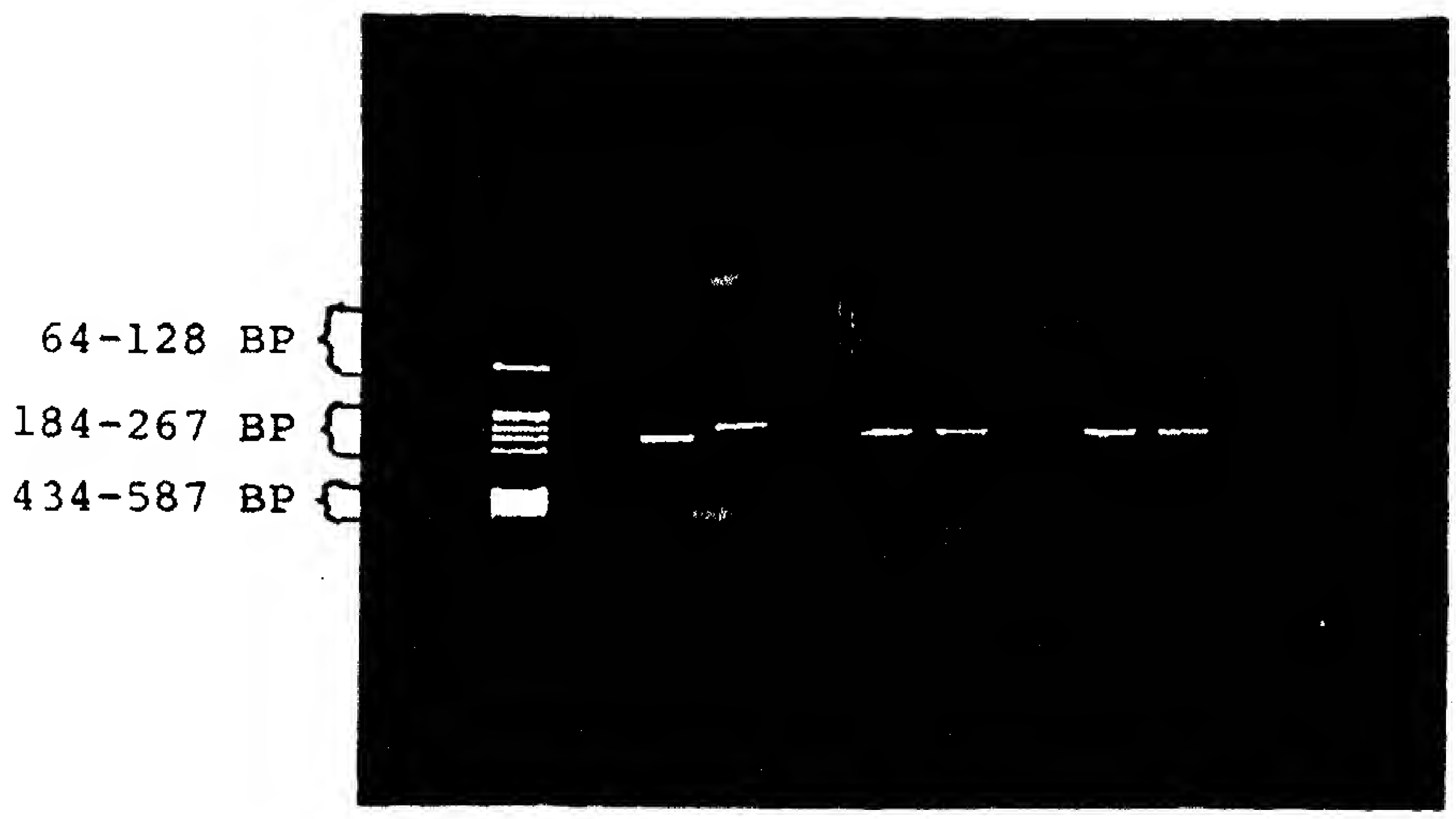
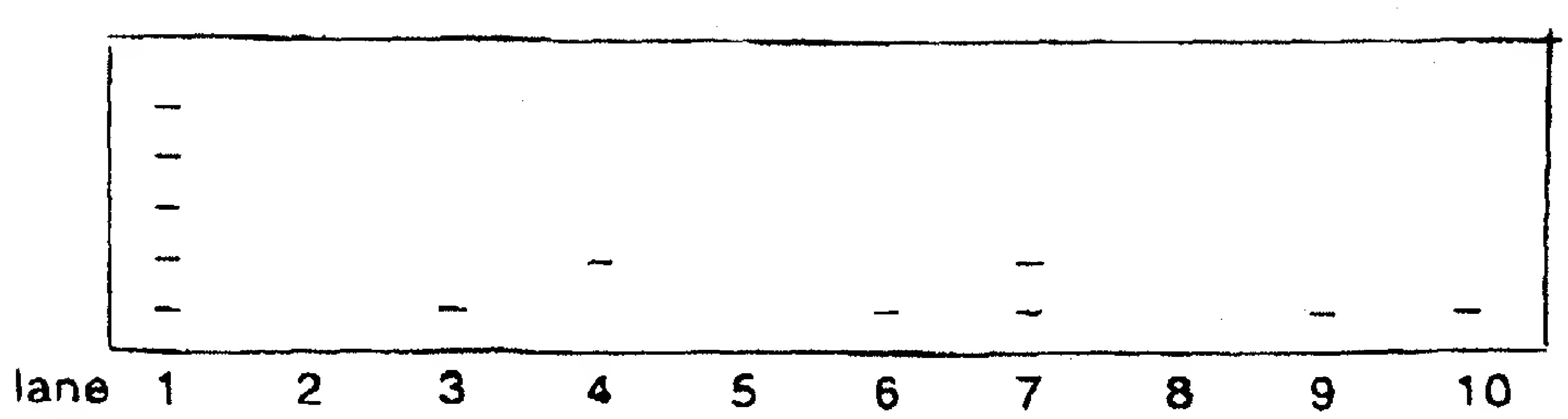


Fig. 4



European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 10 6224

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	LANGDAHL B. L. ET AL.,: "A sequence variation: 713-8delC in the transforming growth factor-beta 1 gene has a higher prevalence in osteoporotic women than in normal women and is associated with very low bone mass in osteoporotic women and increased bone turnover in both osteoporotic and normal women" BONE, vol. 20, no. 3, - March 1997 pages 289-294, XP002076919 * the whole document *	1-17	C12Q1/68
Y	EP 0 593 789 A (SUMITOMO METAL IND) 27 April 1994 * the whole document *	1-17	
A	"GENERATION OF A COMBINATION OF MUTATIONS BY USE OF MULTIPLE MUTAGENIC OLIGONUCLEOTIDES" BIOTECHNIQUES, vol. 20, no. 3, March 1996, page 352, 354 XP002043103 * the whole document *	1-17	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12Q
A	DERYNCK R. ET AL.,: "Intron-exon structure of the human transforming growth factor-beta precursor gene" NUCLEIC ACIDS RESEARCH, vol. 15, no. 7, - 1987 pages 3188-3189, XP002076920 * the whole document * & DATABASE EMBL "AC: X05844"	1-17	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	9 September 1998	Müller, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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(54) Materials and methods for screening anti-osteoporosis agents.

(57) The present invention relates to methods for the identification of therapeutic agents for the treatment of osteoporosis and serum lipid lowering agents. The invention relates to isolating cloning, and using nucleic acids from the promoter regions of transforming growth factor β genes comprising novel regulatory elements designated "raloxifene responsive elements". The invention also encompasses eukaryotic cells containing such raloxifene responsive elements operably linked to reporter genes such that the raloxifene responsive elements modulate the transcription of the reporter genes. The invention provides methods for identifying anti-osteoporosis agents that induce transcription of genes operably linked to such raloxifene responsive elements without inducing deleterious or undesirable side effects associated with current anti-osteoporosis therapy regimens.

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The invention relates to methods for identifying therapeutic agents for the treatment of osteoporosis. The invention relates to isolating, cloning, and using nucleic acids comprising the promoter regions of mammalian transforming growth factor β genes that are novel regulatory elements designated "raloxifene responsive elements". The invention also encompasses genetically engineered eukaryotic cells containing the recombinant expression constructs wherein the raloxifene responsive elements are operably linked to reporter genes. In such cells the raloxifene responsive elements are capable of modulating transcription of the reporter genes in response to treatment with certain compounds. The invention also relates to methods for identifying anti-osteoporosis agents that induce transcription of certain genes via raloxifene responsive elements and that specifically do not induce deleterious or undesirable side effects that have been associated with estrogen replacement therapy, such as increased risk with uterine and breast cancer. The nucleic acids, cells, and methods of the invention provide effective methods for screening putative sources of anti-osteoporosis agents, and identifying those that advantageously lack the undesirable side effects associated with current anti-osteoporosis agents. The invention also relates to a method for inducing bone formation, a method for treating osteoporosis, and a method for treating bone fractures which comprise administering a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element.

In 1991, U.S. pharmaceutical companies spent an estimated $7.9 billion on research and development devoted to identifying new therapeutic agents (Pharmaceutical Manufacturer's Association). The magnitude of this amount is due, in part, to the fact that the hundreds, if not thousands, of chemical compounds must be tested in order to identify a single effective therapeutic agent that does not engender unacceptable levels of undesirable or deleterious side effects. There is an increasing demand for economical methods of testing large numbers of testing large number of chemical compounds to quickly identify those compounds that are likely to be effective in treating disease. At present, few such economical systems exist.

One disease that conspicuously lacks a rapid method for screening large numbers of potential therapeutic agents is bone loss. Bone loss occurs in a wide variety of patients, including those who have undergone hysterectomy, who are undergoing or have undergone long-term administration of corticosteroids, who suffer from Cushing's syndrome or have gonadal dysgenesis, as well as post-menopausal women.

The mechanism of bone loss is not well understood, but in practical effect, the disorder arises from an imbalance in the formation of new healthy bone and the resorption of old bone, skewed toward a net loss of bone tissue. This bone loss includes a decrease in both mineral content and protein matrix components of the bone, and leads to an increased fracture rate of the femoral bones and bones in the forearm and vertebrae predominantly. These fractures, in turn, lead to an increase in general morbidity, a marked loss of stature and mobility, and in many cases, an increase in mortality resulting from complications.

Unchecked, bone loss can lead to osteoporosis, a major debilitating disease whose predominant feature is the loss of bone *mass* without a reduction in bone *volume* (by decreased density and enlargement of bone spaces), producing porosity and fragility. Osteoporosis among post-menopausal women is one of the most common types of bone disorders, affecting an estimated 20 to 25 million women in the United States alone.

A significant feature of post-menopausal osteoporosis is the large and rapid loss of bone mass due to the cessation of estrogen production by the ovaries. Indeed, data clearly support the ability of estrogens to limit the progression of osteoporotic bone loss, and estrogen replacement is a recognized treatment for post-menopausal osteoporosis in the United States and many other countries.

Estrogens, when administered at low levels, have beneficial effects on bone; however, long-term estrogen replacement therapy has been implicated in a variety of disorders, including an increased risk of uterine and breast cancer. These serious side effects cause many women to refuse this treatment. Alternative therapeutic regimens, designed to lessen the cancer risk, such as administering combinations of progestogen and estrogen, cause some patients to experience regular withdrawal bleeding, which is unacceptable to most older women. Concerns over these significant undesirable side effects associated with estrogen replacement therapy, and the limited ability of estrogens to *reverse* existing bone loss, provide a strong impetus for the development of effective alternative therapies for bone loss that do not cause undesirable side effects.

Another approach in osteoporotic therapy is the use of antiestrogens. In general, antiestrogens inhibit (antagonize) the activity of estrogen in the body. Antiestrogens bind to the estrogen receptor, although it is believed that the interaction between antiestrogens and the estrogen receptor involves a different domain of the receptor than that to which estrogen binds. Some antiestrogens, on the other hand, display pharmacological properties that are a mixture of agonist and antagonist properties. In other words, these compounds cause certain effects that mimic estrogen, while antagonizing other effects that are commonly associated with estrogen administration in cells that express the receptor. Because of this mixed effect of some antiestrogens, they are subject to the same adverse effects associated with estrogen replacement therapy.

One antiestrogen known to display such a mixed agonist/antagonist effect is tamoxifen, a drug used for the treatment of breast cancer. Tamoxifen acts as an estrogen antagonist in its ability to reduce the growth

of breast tumors, but it also acts as an agonist in its ability to reduce the amount of serum cholesterol in both healthy women and women with breast cancer. Love *et al.*, *Annals Int. Med.*, **115**, 860-864 (1991). Tamoxifen also act to increase bone density in breast cancer patients. Love *et al.*, *N. Eng. J. Med.*, **326**, 852-856 (1991). At least one study has suggested that the increases in bone density possible with tamoxifen appear to be restricted to the lumbar spine, with bone *loss* being reported in the radius in some breast cancer patients treated with tamoxifen. Furthermore, tamoxifen treatment has also been suggested to contribute to weight gain among post-menopausal women. Love *et al.*, *Ann. Int. Med.*, *ibid.*

Improved anti-osteoporotics that achieve increases in bone density without causing negative side effects are clearly needed. Unfortunately, no method currently exists for rapidly and efficiently screening large numbers of compounds to identify those that display the desired anti-osteoporotic effects. Because this screening process comprises the most time-consuming and expensive step in identifying improved anti-osteoporotic compounds, development of a rapid method for testing large numbers of compounds to identify those that are likely to possess anti-osteoporotic effect is highly desirable.

It is well established that estrogens exert their effects by first binding to an estrogen receptor, and then the estrogen/estrogen receptor complex binding to DNA. The hormone/receptor complex modulates gene expression *via* this DNA binding. Kumar, *Cell*, **55**, 145-156 (1988). Antiestrogens also bind to estrogen receptors. Although these antiestrogen/receptor complexes bind to DNA they generally fail to modulate gene expression. Both estradiol/estrogen receptor complexes and hydroxytamixofen/estrogen receptor complexes bind *in vitro* to DNA binding domains called estrogen responsive elements. Kumar, *Cell*, *ibid.*

The conformation of the ligand/receptor complex is a matter of some debate. However, recent studies have suggested a conformational difference between estrogen receptor bound to estradiol and the same estrogen receptor bound to 4-hydroxytamoxifen or ICI 164,384. Klinge *et al.*, *J. Ster. Biochem. Mol. Biol.*, **43**, 249-262 (1992).

In an effort to rationally address the problem of developing improved anti-osteoporotic agents, researchers have investigated proteins known to play a role in bone maintenance. One protein known to influence bone remodelling and bone turnover is transforming growth factor β (TGFβ). Although commonly referred to as a single compound, TGFβ is actually a family of molecules that now is known to include at least three isoforms: TGFβ-1, TGFβ-2 and TGFβ-3. *See* Arrick *et al.*, *Canc. Res.*, **50**, 299-303 (1990). The present inventor has noted that ovariectomy induces a significant decrease in TGFβ-3 in rat bone (data collected by present inventor is unpublished); others have noted the same type of correlation with respect to levels of TGFβ (isoform not specified). *See* Finkelman *et al.*, *Proc. Nat'l Acad. Sci. USA*, **89**, 12190-12193 (1992). Further, the present inventor has noted that administration of raloxifene, an antiestrogen, to ovariectomized rats restores TGFβ-3 concentrations to levels equal to or higher than that found in control animals. The direct correlation between TGFβ-3 levels and circulating levels of estrogen or antiestrogen, and the finding that TGFβ (isoform not specified) plays a significant role in bone remodelling and turnover, suggest that osteoporosis may result from reduced expression of TGFβ-3 in vivo. *See* Noda *et al.*, *Endocrin.* **12**, 2991-2994 (1989).

The hypothesis that reduced levels of TGFβ-3 may allow bone loss is undermined by the findings that TGFβ has been isolated from a large number of sources and exhibits widely divergent effects. For example, it inhibits the growth of mesenchymal cells and epithelial cells, it induces biosynthesis of proteoglycans, fibronectins, and plasminogen activator, and is chemotactic for fibroblasts, macrophages, and smooth muscle cells. *See*, Flaumenhaft *et al.*, *J. Cell. Bio.*, **120**(4), 995-1002 (1993).

Furthermore, antiestrogens such as tamoxifen or toremifene induce human fetal fibroblasts to secrete TGFβ (without reference to isoform) in the absence of estrogen receptor Colletta *et al.*, *Br. J. Cancer*, **62**, 405-409 (1990). TGFβ has been found to stimulate osteoblastic bone formation and to inhibit osteoclast formation and osteoclast activities. Mundy, "Clinical Application of TGFβ", **Ciba Foundation Symposium No. 157**, 137-151, Wiley, Chichester. TGFβ repressed division of one human endometrial cancer cell line (Ishikawa), but was shown to be mitogenic with respect to another such cell line (HEC-50). Murphy *et al.*, *J. Ster. Biochem. Molec. Bio.*, **41**, 309-314 (1992).

Three months of antiestrogen treatment with tamoxifen has been correlated with induction of extracellular TGFβ-1 in breast cancer biopsies. Butta *et al.*, *Cancer Res.*, **52**, 4261-4264 (1992). Decreased concentrations of TGFβ-1 mRNA were found in one human endometrial cancer cell line (HEC-50) grown in media containing 1% ctFBS (twice charcoal stripped FBS) when such cells were exposed to either estradiol or certain antiestrogens. Gong *et al.*, *Canc. Res.*, **52**, 1704-1709 (1992).

TGFβ-2 mRNA is expressed by the T-47D and MDA-MB-231 cell lines. Treatment of these cell lines with estradiol reduced TGFβ-2 mRNA expression, but tamoxifen did not exhibit the same effect. TGFβ-3 induces mitogenesis, collagen synthesis, and alkaline phosphatase activity in osteoblast enriched bone cell cultures at a three to five fold higher rate than TGFβ-1. Arrick et al., *Canc. Res.*, **50**, 299-303 (1990).

A general review of the properties of TGFβ are described in Sporn *et al.*, *J. Cell Bio.*, **105**, 1039-1045 (1987);

Massague, *Cell*, **49**, 437-438 (1987); and Moses, *Cell*, **63**, 245-247 (1990). These references generally describe the properties exerted by TGFβ in *in vitro* and *in vivo* systems.

The complex pattern of expression described above suggests a unique and complex mechanism of regulation of expression of the various TGFβ isoforms. The promoter regions for each of the genes TGFβ-1, TGFβ-2 and TGFβ-3 have been cloned and described. Kim *et al.*, *J. Biol. Chem.*, **264**, 402-408 (1989); Noma *et al.*, *Growth Factors*, **4**, 247-255 (1991); Lafyatis, *et al.*, *J. Biol. Chem.*, **265**, 19128-19136 (1990).

The promoters for TGFβ-2 and TGFβ-3 have been characterized and have been reported to contain cAMP responsive elements, AP-1 sites, AP-2 sites, and SP-1 sites. Noma *et al.* indicated that the TGFβ-2 promoter activity was dependent upon the region of the promoter investigated and the cell line selected for the induction assay.

At least one method for efficiently screening the biological activity of a large number of compounds is described in International Patent Application No. PCT/US92/00419, which claims methods for transcriptionally regulating the expression of a growth factor. This patent disclosed assays to identify compounds capable of inducing transcription via the promoter regions of the human growth hormone gene, the c-ErbB2 gene, the promoter region of the K-ras sequence, and the early promoter and enhancers of cytomegalovirus. This application is directed primarily towards the problem of determining the regulation of various oncogenes.

To date, identifying compounds that are likely to display an anti-osteoporotic effect has required virtually random investigation of individual compounds on the basis of epidemiological studies, the utility of related chemical compounds in achieving the desired effect, and other time consuming and inefficient methods. Both the delay caused by the current screening methods and the economic costs of such inefficient testing emphasize the need for economical and efficient methods for identifying potential anti-osteoporotic drugs worthy of additional investigation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the promoter region of the TGFβ-1 gene.

Figure 2 depicts the promoter region of the TGFβ-2 gene.

Figure 3 depicts the promoter region of the TGFβ-3 gene.

Figure 4 depicts the relative levels of expression of reporter gene in cells transfected with expression constructs comprising the TGFβ-1 promoter operably linked to the CAT gene, the TGFβ-2 promoter operably linked to the CAT gene, or the TGFβ-3 promoter operably linked to the CAT gene, in the presence of a control compound, estrogen, raloxifene, and tamoxifen. In general, the TGFβ-3 construct containing-cells express the highest levels of reporter gene, followed by those containing the TGFβ-2 construct and the TGFβ-1 construct.

Figure 5 depicts the induction of a reporter gene under the control of either the estrogen responsive element or a portion of the TGFβ-3 promoter in the presence of estrogen, raloxifene, and combinations of estrogen and raloxifene. This figure shows the markedly different patterns of regulation exerted by the two regulatory sequences.

Figure 6 is a bar graph showing the relative level of reporter gene expression in cells transfected with expression constructs containing a portion of the TGFβ-3 promoter sequence and exposed to a control compound, estrogen, raloxifene, and combinations of estrogen and raloxifene in both the presence and absence of estrogen receptor. Estrogen receptor is necessary for induction of expression of the reporter gene by both estrogens and antiestrogens.

Figure 7 depicts the various domains of estrogen receptor (ER) protein expressed by the deletion constructs set forth in Example XI. Additionally, the relative fold induction achievable in cells transfected with the various mutant ERs and an expression construct comprising the TGFβ-3 promoter and the luciferase gene are shown.

Figure 8 depicts the levels of reporter gene expression achievable in cells transfected with an expression construct comprising the TGFβ-3 promoter and the CAT gene in the presence of various concentrations of estradiol, raloxifene, tamoxifen, and ICI 164,384. In general, raloxifene is the most potent inducer at all concentrations, followed by ICI 164,384, tamoxifen. Estrogen is the least potent inducer in this system.

Figure 9 depicts the relative expression of reporter gene in CHO cells transfected with an expression construct comprising a portion of the TGFβ-3 promoter and the luciferase gene in the presence of various concentrations of estradiol and raloxifene. In general, raloxifene is the more potent inducer except at low concentrations.

Figure 10 depicts the relative expression of reporter gene in MCF-7 cells transfected with an expression construct comprising the TGFβ-3 promoter and the luciferase gene in the presence of various concentrations of estradiol and raloxifene. Raloxifene is the more potent inducer at all concentrations.

Figure 11 represents the chemical structures of the compounds evaluated in Example X.

Figure 12 represents the relative level of induction of reporter gene expression in MG63 cells transfected with TGFβ-3 promoter/CAT expression constructs and exposed to various concentrations of the compounds set forth in Example X. Overall, compound 177366 is the most potent inducer, while 98005 shows no induction.

Figure 13 depicts the portions of the TGFβ-3 promoter used to identify the 41 base pair raloxifene responsive element, and depicts the relative induction of reporter gene expression by raloxifene in cells transformed with plasmids comprising the indicated portion of the TGFβ-3 promoter sequence operably linked to a reporter gene. Although the fold induction achievable with the pB-301 construct is highest, presence of the raloxifene responsive element (base pairs +35 - +75) is clearly essential for any significant induction of transcription by raloxifene in these cells.

Figure 14 depicts an analysis of the TGFβ-3 promoter. The major transcriptional start site and a CCCTC-motif are depicted as described in Example XI.

Figure 15 depicts the relative expression of reporter gene in Hep2 cells transfected with an expression construct comprising the LDLR promoter and the luciferase gene in the presence of estrogen receptor and various concentrations of estradiol and raloxifene. Generally, raloxifene is the more potent inducer.

Figure 16 depicts the relative expression of reporter gene in Hep2 cells transfected with an expression construct comprising the LDLR promoter and the luciferase gene in the presence of various concentrations of estradiol and raloxifene and in the absence of estrogen receptor. No induction is exhibited by either compound at concentrations at or below 10^{-6} M. High concentrations of raloxifene induce expression somewhat, suggesting an alternate, non-ER dependent induction mechanism at such concentrations.

Figure 17 is a flow diagram showing an example of a sequence of steps that can be carried out according to the teachings of the present invention to evaluate compounds with respect to their ability to induce transcription of reporter genes operably linked to the regulatory control element described herein. It is expected that a correlation will exist between compounds showing the induction profiles described in Example XIII and the ability of such compounds to act as anti-osteoporosis drugs *in vivo.*

According to the present invention, there is provided novel and efficient methods for screening chemical compounds to determine whether these compounds are capable of modulating steroid hormone-responsive gene expression from a promoter comprising a raloxifene responsive element. There is also provided nucleic acids consisting essentially of a nucleotide sequence comprising a raloxifene responsive element isolated from the promoter region of a TGFβ gene, and eukaryotic cells transfected therewith. There is also provided a recombinant expression construct comprising the raloxifene responsive element operably linked to a reporter gene. There is also provided a method for inducing bone formation, a method for treating osteoporosis, and a method for treating bone fractures which comprise administering a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ gene.

The present invention relates to novel and efficient methods of screening chemical compounds to determine whether those compounds are capable of modulating steroid hormone-responsive gene expression from a mammalian promoter comprising a raloxifene responsive element as discovered and described herein. The invention comprises nucleic acids consisting essentially of the nucleotide sequence of a mammalian promoter comprising such a raloxifene responsive element. In a preferred embodiment, the promoter comprising the raloxifene responsive element is derived from the promoter region of the gene for human TGFβ-3 or TGFβ-2.

The invention further comprises recombinant eukaryotic expression constructs comprising a promoter having a raloxifene responsive element that is operably linked to a reporter gene. In preferred embodiments, the reporter gene is the chloramphenicol acetyltransferase gene or the luciferase gene. Particularly, preferred is the luceferase gene. Cells transfected with such eukaryotic expression constructs, that are capable of expressing the reporter gene when such cells are exposed to raloxifene or other anti-estrogenic compounds, are also provided by the invention.

The present invention further comprises a method for inducing bone formation which comprises administering an effective amount of a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene. The present invention also includes a method for treating osteoporosis which comprises administering an effective amount of a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene. The present invention also provides a method for treating bone fractures which comprises administering an effective amount of a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene.

In the first aspect, the invention provides a nucleic acid consisting essentially of a nucleotide sequence comprising a raloxifene responsive element, where the element is isolated from the promoter region of a mammalian, preferably human, transforming growth factor β gene. In preferred embodiments, the transforming

growth factor β gene is the human TGFβ-2 gene or the human TGFβ-3 gene. In further preferred embodiments of this aspect of the invention, the nucleic acid consists essentially of promoter sequences of the TGFβ-3 gene as described in plasmids pB-301 (containing TGFβ-3 promoter sequences from positions -301 to +110); pB-221 (containing TGFβ-3 promoter sequences from positions -221 to +110); pB-91 (containing TGFβ-3 promoter sequences from positions -91 to +110); pB-60 (containing TGFβ-3 promoter sequence from positions -60 to +110); pB-47 (containing TGFβ-3 promoter sequence from positions -47 to +110) and pB-38 (containing TGFβ-3 promoter sequence from positions -38 to +110), as further described herein.

In a second aspect, the invention provides a recombinant expression construct comprising a nucleic acid consisting essentially of a nucleotide sequence comprising a raloxifene responsive element, where the element is isolated from the promoter region of a mammalian, preferably human, transforming growth factor β gene, operably linked to a reporter gene. In preferred embodiments, the transforming growth factor β gene is the human TGFβ-2 gene or the human TGFβ-3 gene. In preferred embodiments, the reporter gene is the chloramphenicol acetyltransferase gene or the luciferase gene. Particularly, preferred is the luciferase gene. In further preferred embodiments of this aspect of the invention, the nucleic acid comprises a promoter sequence consisting essentially of the promoter sequences of the TGFβ-3 gene comprising the plasmids pB-301 (containing TGFβ-3 promoter sequence from positions -301 to +110); pB-221 (containing TGFβ-3 promoter sequences from positions -221 to +110); pB-91 (containing TGFβ-3 promoter sequences from positions -91 to +110); pB-60 (containing TGFβ-3 promoter sequences from positions -60 to +110); pB-47 (containing TGFβ-3 promoter sequence from positions -47 to +110) and pB-38 (containing TGFβ-3 promoter sequence from positions -38 to +110), as further described herein and operably linked to a reporter gene.

In another aspect, the recombinant expression constructs of the invention are capable of expressing the reporter gene encoded by such a construct in eukaryotic cells transfected with such a construct. In preferred embodiments, such eukaryotic cells additionally express an estrogen receptor protein or mutant derivative thereof. In particularly preferred embodiments, expression of the reporter gene by the recombinant expression constructs of the invention is capable of being induced by treatment of such cells with raloxifene or other anti-estrogenic compounds as defined herein.

A third aspect of the invention provides a eukaryotic cell into which has been introduced a recombinant expression construct of the invention. In a preferred embodiment, the eukaryotic cell is a cell transfected with a recombinant expression construct of the invention. In a preferred embodiment, the eukaryotic cells of the invention express an estrogen receptor protein or mutant derivative thereof. In particularly preferred embodiments, expression of the reporter gene in such cells is capable of being induced by treatment of such cells with raloxifene or other anti-estrogenic compounds as defined herein.

The invention also provides methods for screening a multiplicity of compounds to identify those compounds having potential as anti-osteoporotic agents. In one aspect of this embodiment of the invention is provided a method for screening a multiplicity of compounds to identify compounds having potential as anti-osteoporosis agents. The method provided by this aspect of the invention comprises identifying a compound of the multiplicity that is capable of inducing transcription from a raloxifene-responsive element of a mammalian promoter, is a specific transcription inducer, is not capable of inducing transcription from an estrogen-responsive element of a mammalian promoter, and is an anti-estrogenic or non-estrogenic compound. The method provided by this embodiment further comprises the steps of (a) assaying for the ability of the compound to induce transcription from a raloxifene responsive element of a mammalian promoter; (b) assaying for the inability of the compound to induce transcription from a mammalian promoter not having a raloxifene responsive element; (c) assaying for the inability of the compound to induce transcription from an estrogen responsive promoter; and (d) assaying for the ability of the compound to inhibit estrogen induction of transcription from an estrogen responsive promoter in the presence of estrogen.

In a preferred embodiment, the assay of subpart (a) comprises the step of determining the ability of the compound to induce expression of a reporter gene operably linked to the mammalian promoter comprising a raloxifene responsive element. In another preferred embodiment, the assay of subpart (b) comprises the step of determining the inability of the compound to induce expression of a reporter gene operably linked to the mammalian promoter wherein the promoter is not comprised of a raloxifene-responsive element. Another preferred embodiment of this aspect of the invention provides the assay of subpart (c) comprising the step of determining the inability of the compound to induce expression of a reporter gene operably linked to an estrogen responsive mammalian promoter. In a final preferred embodiment, the invention provides the assay of subpart (d) comprising the step of determining the ability of the compound to inhibit estrogen-dependent induction of expression of a reporter gene operably linked to an estrogen responsive mammalian promoter in the presence of estrogen.

In particularly preferred embodiments of this aspect of the invention, the raloxifene responsive mammalian promoter is isolated from a mammalian, preferably human, transforming growth factor β gene. Most preferred

are embodiments wherein the transforming growth factor β gene is the human TGFβ-2 gene or the human TGFβ-3 gene. In other preferred embodiments, the reporter gene is the chloramphenicol acetyltransferase gene or the luciferase gene. In further preferred embodiments of this aspect of the invention, the raloxifene responsive promoter sequences consisting essentially of the promoter sequences of the TGFβ-3 gene comprising the plasmids pB-301 (containing TGFβ-3 promoter sequences from positions -301 to +110); pB-221 (containing TGFβ-3 promoter sequences from positions -221 to +110); pB-91 (containing TGFβ-3 promoter sequences from positions -91 to +110); pB-60 (containing TGFβ-3 promoter sequences from positions -60 to +110); pB-47 (containing TGFβ-3 promoter sequences from positions -47 to +110) and pB-38 (containing TGFβ-3 promoter sequences from positions -38 to +110), as further described herein and operably linked to a reporter gene.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

This specification contains a number of abbreviations. As used herein, "TGFβ" shall mean transforming growth factor β (without reference to isoform). TGFβ-1, TGFβ-2 and TGFβ-3 shall have the meanings well-established in this art, i.e., to represent the three known isoforms of transforming growth factors β genes. As used herein, the abbreviation "CAT" shall be taken to mean chloramphenicol acetyl CoA transferase. "Estradiol" is an estrogen and, at times, is abbreviated herein as E2. As used herein, the abbreviation "ER" shall mean an estrogen receptor protein.

The term "raloxifene responsive element" as used herein refers to nucleotide sequences of the nucleic acid comprising a mammalian promoter region of the TGFβ gene that are capable of inducing transcription of any structural gene to which the raloxifene responsive element is operably linked in host cells that are exposed to raloxifene and estrogen receptor proteins. Raloxifene responsive elements include, but are not limited to the nucleotide sequences comprising the TGFβ promoter sequences of the plasmids pB-301 (containing TGFβ-3 promoter sequences from positions -301 to +110); pB-221 (containing TGFβ-3 promoter sequences from positions -221 to +110); pB-91 (containing TGFβ-3 promoter sequences from positions -91 to +110); pB-60 (containing TGFβ-3 promoter sequences from positions -60 to +110); pB-47 (containing TGFβ-3 promoter sequences from positions -47 to +110) and pB-38 (containing TGFβ-3 promoter sequences from positions -38 to +110), as further described herein, and nucleic acids having substantially the same biological activity as those nucleic acids. This definition is intended to encompass natural allelic variations in the promoter regions of the TGFβ genes. Isolated raloxifene responsive elements of the present invention may be derived from TGFβ promoters of any mammalian species of origin, but are preferably of human origin.

As used herein, anti-estrogens will be taken to include full and partial antagonists of estrogen. All estradiols used in the Examples described herein are 17β-estradiol.

The term "effective amount" represents an amount of a compound that, when bound to an estrogen receptor, is capable of inducing transcription from a raloxifene responsive element when administered to a mammal. The particular dose of compound administered will be determined by the particular circumstances surrounding the case, including the compound administered, the route of administration, the particular condition being treated, and similar considerations.

The term "potently" represents a compound that, when bound to an estrogen receptor, induces transcription from a raloxifene responsive element at a minimum effective concentration (MEC) of less than or equal to 10nM ($1x10^{-8}$M), when the compound is tested in the *in vitro* assay described herein. *See* Examples V, VI, X, and XIV.

All portions of promoter sequences are identified in terms of their distance, in number of nucleotides, from the major transcriptional start site of the gene, taking this start site to be +1 as shown in Figures 1-3. A negative sign (-) preceding the number indicates the nucleotide is 5' to the start site, a positive sign (+) preceding the number indicates the nucleotide is 3' to the start site. The sequences are also identified by the numbering indicated in SEQ ID NOS:1-3, and are specifically correlated with numbering of Figures 1-3.

DNA that encodes the raloxifene responsive elements of the present invention may be obtained, in view of the instant disclosure, by chemical synthesis, by *in vitro* amplification [including but not limited to the polymerase chain reaction (PCR)], or by combinations of these procedures from naturally-occurring sources, such as cultures of mammalian cells, genomic DNA from such cells, or libraries of such DNA.

The raloxifene responsive elements may be advantageously operably linked to reporter genes and used to either transiently or stably transform appropriate host cells through the use of appropriate vectors, constructs, and means well known in the art, such as DNA mediated gene transfer means including but not limited to transfection, electroporation, and virally-mediated infection. The term "recombinant expression construct" as used herein is intended to mean DNA constructs capable of directing the expression of reporter genes to which the raloxifene responsive elements of the invention are operably linked.

DNA regions are operably linked when they are functionally related to each other. For example, a promoter

is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous.

Reporter genes are genes that encode structural proteins capable of quantification by standard means such as measuring enzymatic activity, colorimetry, chemiluminescence, the presence of radioactivity in a sample, ELISA, antibody binding, radioimmunoassay or other methods of quantification known to those in the art. The reporter gene used will vary depending upon the host selected and the transformation method chosen. Useful reporter genes include but are not limited to chloramphenicol acetyltransferase, luciferase, β-galactosidase, alkaline phosphatase, or any other quantifiable protein product. In a preferred embodiment of the invention, the reporter gene is luciferase.

Transfected cells are cells that have been transfected with raloxifene responsive element-reporter gene recombinant expression constructs made using recombinant DNA techniques. Cells that have been transfected with recombinant raloxifene responsive element-reporter gene expression constructs that express the estrogen receptor, either as a characteristic of such cells or due to the co-transfection of an estrogen receptor encoding expression construct, will express the gene product of the reporter gene under appropriate circumstances (i.e., exposure to an anti-estrogen or other inducer). For example, a preferred cell line appropriate for use in the present invention, MCF-7, constitutively expresses the estrogen receptor. For such cell lines, transfection with the recombinant raloxifene responsive element-reporter gene expression constructs alone will yield cells appropriate for use in the present invention. Alternatively, MG63 cells express reporter genes in a raloxifene-dependent manner, only upon cotransfection of a raloxifene responsive element-reporter gene expression construct and an estrogen receptor expression construct.

Cultures of cells derived from multicellular organisms are desirable hosts for expression of the raloxifene responsive element-reporter gene expression construct. In principle, any higher eukaryotic cell culture that either naturally expresses the estrogen receptor, or that has been genetically modified to express the estrogen receptor (or part of that receptor) is useable. Mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. *See* **Tissue Culture**, Academic Press, Kruse & Patterson, editors (1973). Examples of useful host cell lines are MCF-7, MG63, HeLa, RL95.2, HepG2 and CHO cells (all available from the American Type Culture Collection, Rockville, Maryland). For the purposes of the present invention, use of the MCF-7 cell line is particularly preferred, as this cell line constitutively expresses estrogen receptor.

Host cells that express the estrogen receptor or part of that receptor and contain a raloxifene responsive element-reporter gene expression construct can be used to evaluate compounds for their ability to induce transcription via the raloxifene responsive element as described in the Examples *infra*. In a preferred embodiment of the invention, compounds will be considered to induce transcription via a regulatory element (including but not limited to nucleic acid derived from a TGFβ promoter or deletion construct thereof) if transcription of the reporter gene is increased twofold in the presence of the compound compared with expression in the absence of the compound. In a less preferred embodiment, compounds will be considered to be transcriptional inducers if they induce transcription to a level fifty percent above that of the control. In general, however, induction detectably above background is adequate to show induction by a chemical compound.

In the practice of the aspects of the invention embodying screening methods (*see* Example XIII), use of the plasmid pB-301 is preferred due to the high level of responsiveness to raloxifene exhibited by this plasmid. Other embodiments utilize constructs containing the TGFβ-3 promoter region encompassing positions -38 to +75. In all operative embodiments of the invention, the raloxifene responsive element is operably linked to a reporter gene in a context allowing transcription, as this element is necessary to allow the raloxifene responsive induction described herein.

The order of carrying out the steps of the screening methods of the invention may be varied, and in some instances, some of the steps may be omitted. The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLE I

Construction of Reporter Plasmids

A. phTG12, pTGF-1 and pB-499

As a first step in developing cell lines useful in screening potential anti-osteoporotic agents, a series of reporter plasmids encoding the chloramphenicol acetyltransferase gene (CAT) (Gorman *et al*., *Molec. Cell.*

Biol., **2**, 1044-1051 (1982)) operably linked to promoter sequences from the TGFβ-1, TGFβ-2 and TGFβ-3 genes were obtained from A. Roberts, National Institutes of Health, Laboratory of Chemoprevention (NIH/NCI, Bethesda, MD). These plasmids were designated phTG12 (TGFβ-1), pTGF-1 (TGFβ-2) and pB-499 (TGFβ-3), respectively. The sequences for each of these promoters can be found in Kim *et al., J. Biol. Chem.*, **264**, 402-408 (1989) (TGFβ-1); Noma *et al., Growth Factor,* **4**, 247-255 (1991) (TGFβ-2); and Lafyatis *et al., J. Biol. Chem.*, **265**, 19128-19136 (1990) (TGFβ-3). The promoter sequence of the TGF_-1 gene has been submitted to GenBank/EMBL Data Bank under accession number J04431. The TGFβ-1, TGFβ-2 and TGFβ-3 promoter sequences are shown in Figures 1, 2 and 3, respectively, and as SEQ ID NOS: 1, 2 and 3, respectively.

Alternatively, CAT-containing reporter plasmids operably linked to each of the TGFβ promoter sequences can be produced by subcloning each TGFβ promoter into a commercially-available CAT construct, *for example*, pCAT-Basic (Promega, Madison, WI), using conventional cloning techniques. *See* **Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual**, 2d ed., Cold Spring Harbor Press, Cold Spring Harbor, New York (hereinafter **Sambrook et al.**).

In order to identify region(s) of the TGFβ-3 gene promoter responsive to the antiestrogen raloxifene, CAT reporter gene expression directed by constructs containing partial sequences of the TGFβ-3 gene promoter were analyzed. Six TGFβ-3 promoter deletion/CAT reporter constructs were obtained from A. Roberts. Plasmid designations and the extent of the promoter region contained in each of these plasmids are set forth below:

pB-301	-301 to +110	(corresponding to 1896 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-221	-221 to +110	(corresponding to 1976 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-91	-91 to +110	(corresponding to 2106 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-60	-60 to +110	(corresponding to 2137 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-47	-47 to +110	(corresponding to 2150 to 2306 shown in Figure 3 and SEQ ID NO:3)
pB-38	-38 to +110	(corresponding to 2159 to 2306 shown in Figure 3 and SEQ ID NO:3)

Two additional TGFβ-3 promoter deletion constructs were constructed as described below. The first of these consisted of human TGFβ-3 promoter region sequences corresponding to positions -38 to +75 in the promoter sequence, corresponding to 2159 to 2271 shown in Figure 3 and SEQ ID NO:3 (*see* Lafyatis *et al., ibid.*). The second promoter deletion construct consisted of human TGFβ-3 promoter region sequences corresponding to positions -38 to +35 in the promoter sequence, corresponding to 2159 to 2231 shown in Figure 3 and SEQ ID NO:3. The well-established practice in this art is to identify all promoter sequences identified with respect to the distance from the transcription start site.

These plasmids were generated as follows. Oligonucleotides corresponding to the extent of the TGFβ-3 promoter sequence desired in each plasmid were synthesized using a DNA/RNA synthesizer (Model 394, Applied Biosystems Inc., Foster City, CA) under β-cyanoethyl phosphoamidite synthesis conditions specified by the manufacturer. Complementary pairs of oligonucleotides for each plasmid construct were synthesized, purified, mixed, and allowed to anneal to form double-stranded DNA corresponding to the appropriate TGFβ-3 promoter sequences using conventional methods (**Sambrook et al.**, *ibid.*). *Hin*dIII and *Xba*I restriction enzyme recognition sites were synthesized as the appropriate overhanging ends at the 5' and 3' ends of the sequences, respectively. Double-stranded promoter sequences were then ligated into the *Hin*dIII/*Xba*I-digested CAT reporter plasmid pB-301 and propagated in bacteria under standard conditions. The reporter plasmid produced in this way that contained the -38 to +75 region of the TGFβ-3 promoter was termed pTGFβ+75CAT, and the plasmid that contained the -38 to +75 region of the TGFβ-3 promoter was termed pTGFβ+35CAT. These plasmids were used in CAT assays as described below in Example V.

B. Luciferase reporter plasmids containing TGFβ-3 promoter deletion constructs, including control containing no portion of the promoter region: pTGFβ-301LUC, pTGFβ-38LUC, pTGFβ+75LUC, pTGFβ +35LUC and pLUC

Four plasmids were constructed containing the luciferase gene (REF) expressed under the transcriptional control of TGFβ-3 promoter sequences and varying deletion derivatives thereof. The plasmid pTGF-301LUC was made by digesting pB-301 with *Hin*dIII and thereafter the ends of the *Hin*dIII digestion-generated overhang were blunted by treatment with the Klenow fragment of bacterial DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN). *Xba*I digestion was then performed to liberate the portion of the TGFβ-3 promoter correspond-

ing to positions -301 to +110. This fragment was subcloned into *SmaI*/*XbaI*-digested pSP73 (Promega) to generate the shuttle vector pSPTGFβ-301.

After *in vivo* amplification in bacteria, a preparation of isolated and purified pSPTGFβ-301 was digested with *NdeI* and *HindIII*, and the promoter sequence-containing fragment isolated after separation on a 0.8% agarose gel (BRL-LifeTechnologies, Inc., Gaithersburg, MD). The luciferase-containing construct pLDLRLUC10 (as described in U.S. Patent Application Ser. No. 08/018,985, filed March 3, 1993, and further described in Section C., below) was *NdeI*/*HindIII*-digested and purified after agarose gel electrophoresis. These isolated fragments were then mixed, ligated and used to transform bacteria (**Sambrook et al.**, *ibid.*).

The plasmids pTGFβ-38LUC, pTGFβ+75LUC and pTGFβ+35LUC were made by first excising the TGFβ-3 promoter sequences from pB-38, pTGFβ+75CAT and pTGFβ+35CAT, respectively, by *BamHI*/*XbaI* double digestion. The luciferase-containing plasmid pGL2-Basic (or "pGL2LUC") (Promega) was prepared by *NheI*/*BamHI* digestion, and each of the recombinant plasmids made by ligation of the appropriate TGFβ-3 promoter sequences into the luciferase-containing plasmid. These plasmids were used in luciferase assays as described below in Example VI.

A control plasmid containing the luciferase gene but harboring no portion of the TGFβ-3 gene was constructed by digesting pTGFβ+75LUC plasmid DNA with restriction endonuclease *XbaI* and *HindIII*. Protruding ends were filled by Klenow enzyme reaction in the presence of all four dNTPS under standard conditions (**Sambrook et al.**, *ibid.*). The ends thus blunted were ligated with T4 DNA ligase (Boehringer Mannheim) under manufacturer suggested conditions. The resulting plasmid was designated pLUC.

C. LDLR Promoter Containing Reporter Plasmid: pLDLRLUC10

The plasmid pLDLRLUC10 was described in U.S. Patent Appln. Ser. No. 08/018,985, filed March 3, 1993 (hereinafter, the '985 application). The construction of this plasmid is described in detail as follows:

A 1546 base pair sequence of the human LDL receptor promoter was amplified using the polymerase chain reaction.

A reaction mixture containing 20 picomoles each of the synthetic oligonucleotides:

5'-GCGCCATATGAGTCTTAACTGCCAAAAATTCTTATCATCAAT-3'
(SEQ ID NO:4)

and

5'-AAGCAAGCTTTCGCAGCCTCTGCCAGGCAGTGTCCCGACCCGGA-3'

(SEQ ID NO:5)

and 1 μg human genomic DNA purified from the adenocarcinoma cell line P3UCLA, 200 μM each of dATP, dGTP, dCTP and TTP, 2.5 units of *Taq* DNA polymerase, 10mM Tris-HCl pH9.3, 50 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin in a final volume of 100 μL was subjected to 30 cycles consisting of 15 sec at 96°C, 30 sec at 55°C, and 1 min at 72°C. The material was subject to gel electrophoresis on a 1% agarose gel and a 1546 basepair (bp) band isolated and restriction enzyme digested with *HindIII* and *NdeI*. This fragment was ligated into the plasmid pSP72 (Promega), which had previously been digested with *HindIII* and *NdeI*. The resulting vector, pNLDLRP, was digested with *NdeI* and *HindIII*, the material was electrophoresed on a 1% agarose gel and the 1546 base pair LDL receptor sequence reisolated therefrom.

Plasmid vector pSv2 was constructed by digesting plasmid pSv2-globin with *HindIII* and *BglII* then ligating an *NruI*-*XhoI* linker into the vector. Plasmid pSv2 globin is disclosed in U.S. Patent No. 4,775,624, which is incorporated by reference. The linker contained the following sequences:

5'-AGCTTCGCGACTCGAGA-3'
(SEQ ID NO:6) and

```
5'-GATCTCTCCAGTCGCGA-3'
(SEQ ID NO:7).
```

The resulting vector was designated pSv2-H NXB because it contained a *Bam*HI site, an *Nru*I site, an *Xho*I site and a *Bgl*II site. The *Hind*III-*Bgl*II fragment of plasmid pAlc4(NRRL B-18783), which contains the firefly luciferase gene (REF), was then ligated into the *Hind*III-*Bgl*II site of plasmid pSv2-HNXB.

The 1546 base pair fragment described above was isolated and cloned into the vector pSv2 containing firefly luciferase reporter gene that had been restriction enzyme digested to completion with NdeI and partially with *Hind*III. The resulting vector, pLDLRLUC10 contains the human LDL receptor promoter directing expression of the firefly luciferase gene, an ampicillin resistance marker and an origin of replication.

EXAMPLE II

Human Estrogen Receptor Expression Plasmids

The estrogen receptor-containing mammalian expression constructs pCMVER and pRSV-ER were obtained from B.S. Katzenellenbogen, Department of Physiology and Biophysics, University of Illinois (Urbana-Champaign, IL). *See* Reese and Katzenellenbogen, *J. Biol. Chem.*, **266**, 10880-10887 (1990). These plasmids were used in expression assays as described below, *for example*, in Example VII.

EXAMPLE III

Construction of An Estrogen Responsive Element/Luciferase Gene-Containing Plasmid

Complementary oligonucleotides corresponding to the estrogen-responsive element (ERE) from the *Xenopus laevis* vitellogenin *Az* gene promoter, corresponding to positions -341 to -310 [(Metzger *et al.*, *Nature*, **334**, 31-36 (1988)] were designed, synthesized, and annealed to form a double-stranded region that is an estrogen responsive element essentially as described in Example I. A sequence comprising an *Xho*I restriction enzyme recognition site was synthesized to be flanking the ERE sequences, the element having the following nucleotide sequence (shown as SEQ ID NOS:8 and 9, respectively):

```
5'-TCG-AGA-AAA-GTC-AGG-TCA-CAG-TGA-CCT-GAT-CAA-AC-3'
      3'-CT-TTT-CAG-TCC-AGT-GTC-ACT-GGA-CTA-GTT-TGA-GCT-5'
```

The double-stranded ERE was subcloned into *Xho*I-digested pGL12-Basic (Promega), whereby the luciferase gene was placed under the transcriptional influence of the ERE. This plasmid was designated pGL2ERELUC and used in further experiments as described below (Example VI).

EXAMPLE IV

DNA Transfection

A. Cell culture

Mammalian cells were cultured in media (termed 3:1 media) consisting of Dulbecco's modified Eagle's media and F12 media (mixed in a ratio of 3:1, obtained from GIBCO, Grand Island, NY), without phenol red, containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were passaged at four day intervals. One day prior to transfection, cells were trypsinized by incubating them with 1mL of a solution of 0.05% trypsin/5.3mM tetrasodium ethylenediamine tetraacetate (GIBCO) for 5min at room temperature, and then seeded in 3:1 media containing 10% charcoal-stripped FBS (csFBS; Hyclone) at densities of one million cells per 10cm culture dish.

B. Transient co-transfection of TGFβ constructs and human ER constructs

Co-transfection experiments were performed in MG63 (human osteosarcoma) cells, using the ProFection Mammalian Transfection System (Promega). Ten μg of TGFβ promoter-containing reporter plasmid DNA and

5 μg of human estrogen receptor (hER)-containing expression plasmid (pRSV-ER or pCMV-ER, obtained from B.S. Katzenellenbogen, Department of Physiology and Biophysics, University of Illinois [see Reese and Katzenellenbogen, *J. Biol. Chem.*, **266**, 10880-10887 (1990)] or pRSV (control) plasmid were mixed, co-precipitated, and transfected into 2-4 million cells in 10 cm culture dishes. After incubating the cells with the DNA precipitate at 37°C for 15h, the DNA precipitate was removed by twice washing the cells with Dulbecco's phosphate buffered saline (D-PBS; GIBCO). The cells were then refed with fresh 3:1 media containing 10% csFBS. Compounds to be evaluated for their ability to modulate reporter gene expression were added at the appropriate times and assayed as described below.

C. Stable co-transfection of TGFβ constructs and human ER constructs

MCF-7 cells were used for transfection experiments resulting in stable integration of transfected plasmid sequences into the recipient cell genome. In these experiments, TGFβ promoter containing plasmid DNA was mixed with DNA encoding a selectable marker. For example, 60μg of TGFβ promoter-containing plasmid DNA (pTGFβ-301LUC, pGL2LUC or pGL2ERELUC) were mixed with 60μg of the pSV2HYG-derivative, hygromycin resistance gene-containing plasmid pSV2HYGtB (further described in U.S. Patent Application Ser. No. 07/953,633, filed September 29, 1992, hereby incorporated by reference), and transfected onto two million MCF-7 cells using the ProFection system as described above (Promega). After overnight incubation at 37°C, the precipitate was washed from the cells as described above and the cells then refed with fresh 3:1 media containing 10% FBS and cultured for an additional 48h at 37°C, at which time the cells had typically reached confluency. Cells were trypsinized as described above and each culture dish replated into two 10 cm culture dishes in 3:1 media containing 10% FBS. Hygromycin resistance was selected by culturing the cells in media supplemented with 200μg/mL hygromycin B (Calbiochem-Novabiochem, LaJolla, CA). This selective media was replaced with fresh hygromycin B-supplemented media every 2 days without disturbing the cells for the duration of the selection experiment. Clonal colonies became visible after growth for approximately 14 days in selective media. Such clones were isolated and transferred to individual wells of 24-well cell culture dishes (Flow Laboratories, McLean, VA) using a sterile pipette tip. Such clones were grown and maintained in selective media.

To identify hygromycin-resistant clones that had been successfully co-transfected with luciferase gene-containing plasmid sequences, the polymerase chain reaction (PCR) was used to detect luciferase cDNA-derived DNA sequences in transfectant DNA. Oligonucleotide PCR primers were synthesized corresponding to positions 355-373 (sense primer) and 929-911 (antisense primer) of the luciferase cDNA sequence. PCR was performed using Perkin-Elmer GeneAmp PCR System 9600 for 35 cycles under conditions essentially as described by the manufacturer; each PCR cycle included 45 sec at 94°C, 45 sec at 55°C and two minutes at 72°C.

Luciferase-containing hygromycin-resistant clones were incubated with estrogen or raloxifene as described above, and the effect on expression of reporter genes analyzed using assays for the amount on enzymatic activity present in cell extracts. For luciferase assays, cells were lysed in eukaryotic cell lysis reagent containing 0.1M phosphate buffer (pH7.8)/ 1% TritonX-100 (Boehringer Mannheim)/ 2mM EDTA and 1mM dithiothreitol (DTT, Boehringer Mannheim), and assayed using an optimized unenhanced luciferase assay protocol developed by the Analytical Luminescence Laboratory (San Diego, CA). Light output was measured and recorded using a microtitre plate luminometer (ML3000, Dynatech Laboratories, Chantilly, VA). Clones of such cells stably transfected with TGFβ-reporter gene constructs were then used in new anti-osteoporotic screening assays as described below (Example XIII).

EXAMPLE V

Analysis of TGFβ Promoter-Mediated Transcriptional Activation by Estrogen and Antiestrogens

It was known in the prior art that expression of the TGFβ-1, TGFβ-2 and TGFβ-3 genes was differentially inducible using estrogen and tamoxifen. See "Background of the Related Art" above. The extent and pattern of this inducibility was characterized using the TGFβ promoter-containing plasmids described above in a series of *in vitro* expression assays as follows.

Cultures of MG63 cells were transiently co-transfected with pRSVER plasmid and either phTG12, pTGF-1, or pB2-499 using the ProFection system (Promega). For each transfected cell culture, DNA-containing calcium phosphate precipitates were added dropwise to each culture dish and mixed thoroughly in the media. The pH of the media was carefully maintained between pH 7.2 and pH 7.4. Transfected cell cultures were then incubated overnight in a 5% CO_2 atmosphere at 37°C. For all cultures, the precipitate was removed after over-

night incubation by aspirating the media from the culture dishes, followed by rinsing each dish twice with D-PBS. For each co-transfected cell line, the buffer was replaced with 10 ml fresh medium containing 10% csFBS and one of the following compositions:

a. 10 µl ethanol (hormone vehicle) = control;
b. 10 µl 17β-estradiol (Sigma) ("estradiol") at a concentration of 10^{-4}M in ethanol;
c. 10 µl raloxifene (Eli Lilly Laboratories) at a concentration of 10^{-4}M in ethanol;
d. 10 µl Tamoxifen (Sigma) at a concentration of 10^{-4}M in ethanol.

After 24h, incubation with these hormonal preparations (or the vehicle control), cells were washed twice with D-PBS. The cells were then scraped from the culture dishes using a rubber policeman and 1 ml of D-PBS. Cells were collected by centrifugation at 8,000 rpm for two minutes in a tabletop centrifuge (MicroMax Model, IEC, Newark, NY). The supernatant was removed and the cell pellets were resuspended in 150µL of a 0.25M Tris-HCl solution (pH 7.8). Cells were lysed by three cycles of freezing in a dry ice/ethanol bath and thawing in a water bath at 37°C water bath (for 3 minutes each cycle). Lysed cell preparations were centrifuged at 15,000 rpm for 5 minutes at 4°C to remove cell debris. Supernatants containing the soluble cell lysate were transferred to a new set of tubes for assaying chloramphenicol acetyltransferase (CAT) activity.

Before performing CAT assays on such cell lysates, the protein content of each lysate was first determined using a commercially-available assay (BioRad Laboratories, Richmond, CA). An amount of each cell lysate containing 100µg total protein was then mixed with CAT assay buffer (0.4M Tris-HCl (pH 7.8)/ 0.5mM acetyl-CoA (Boehringer Mannheim)/ 0.1 µCi D-*threo*-(dichloroacetyl-1,2-[^{14}C]-chloramphenicol) for 15 hours. After this incubation, reactions were stopped by vigorously extracting the reaction mixture with 900µL ethyl acetate. The organic and aqueous phases were separated by brief centrifugation at 14,000 rpm for 1 minute, and approximately 800µL of the organic phase was transferred to a new set of tubes. Ethyl acetate was evaporated to concentrate the CAT-catalyzed reaction products.

Acetylated and unacetylated chloramphenicol species were resolved by thin layer chromatography using a mixture of 95:5 (v/v) chloroform/methanol as the ascending buffer. Radioactivity from each species so resolved was measured using a Betascope 603 blot analyzer (Betagen, Intelligenetics Inc., Mountain View, CA). The percentage of acetylated counts relative to the total counts was calculated to yield relative CAT activities of each transfectant assayed (all CAT activities expressed herein were calculated on this basis). Each assay was performed in duplicate.

A representation of the results of the above experiment for MG63 transfectant cell lines is shown in Figure 4. The results of a representative experiment are tabulated below:

TABLE I

Promoter	Control	Estradiol		Raloxifene		Tamoxifen	
		Act.	Fold Ind.†	Act.	Fold Ind.	Act.	Fold Ind.
TGFβ-1	6.4	4.7	0.7	5.6	0.9	6.9	1.1
TGFβ-2	0.8	1.29	1.6	2.3	2.9	2.0	2.6
TGFβ-3	0.7	2.1	2.8	5.2	7.3	1.9	2.6

† - Fold induction is calculated based on comparison with control

These experiments demonstrate that transcription of the CAT reporter gene is induced by estrogen and the antiestrogens raloxifene and tamoxifen, with raloxifene displaying a greater potency than estrogen, especially for the TGFβ-3 promoter. In contrast, the TGFβ-1 promoter region used in this experiment (positions -1032 to +727) showed no response to either estradiol or raloxifene.

EXAMPLE VI

Differential Induction of TGFβ-3 Promoter and the ERE of the Vitellogenin Promoter by Estrogen and Raloxifene

The results obtained in the previous Example demonstrated that the TGFβ-3 gene promoter is transcriptionally responsive to both estrogen and "antiestrogen" compounds such as raloxifene and tamoxifen, and that transcription was induced by raloxifene treatment to a relatively greater degree than the degree of transcriptional induction produced in response to estrogen. This example demonstrates that the gene encoding the *Xenopus* protein vitellogenin responds *in vivo* in exactly the opposite fashion, i.e., transcription of the vitellogenin gene is strongly induced by estrogen and only weakly induced by raloxifene. In addition, raloxifene strongly antagonizes estrogen-induced induction of vitellogenin production when the two compounds are given together. The instant results suggested that the TGF_ promoter sequences directing transcription of the reporter genes in the reporter plasmids assayed above in Example VI contain a novel raloxifene-responsive element, characterized by a unique pattern of estrogen and antiestrogen responsiveness.

This pattern of estrogen and antiestrogen responsiveness was further investigated using the reporter gene assay system described in previous Examples. Cultures of MG63 cells were transiently co-transfected with pB-301 and pRSVER as described in Example IV. Such transiently transfected cultures were tested for transcriptional activation of reporter gene expression by treatment with estradiol and raloxifene at concentrations varying in ten-fold increments from 10^{-9}M to 10^{-5}M. The combination of estrogen and raloxifene was also tested by assaying the effects of raloxifene at 10^{-8}M on reporter gene induction in response to estrogen using the same series of concentrations as with estrogen alone. These assays were performed essentially as in Example V.

Similar assays were performed using cultures of MG63 cells transiently transfected with pGL2ERELUC and pRSVER. In these assays, however, the amount of raloxifene added in combination with estrogen was varied so that the raloxifene concentration was twenty times the concentration of estrogen in the mixture (i.e., 10^{-9}M estrogen/2 x 10^{-8}M raloxifene; 10^{-8}M estrogen/ 2 x 10^{-7}M raloxifene, etc.).

Transfected cells were treated with varying amounts and combinations of hormones and then rinsed twice with D-PBS. Cells were then lysed upon incubation with 250μL of eukaryotic cell lysis reagent (as described in Example VI) at 4°C for 20 min and transferred to microcentrifuge tubes by scraping with a rubber policeman. Cell lysates were centrifuged at 14,000 rpm for one minute to remove cell debris. Cell extracts (supernatant) were then assayed for protein content and luciferase activities.

Luciferase assays were performed as follows. 50μL of each cell extract was added to 100μL of reagent A buffer (containing 4.0mM ATP/15mM $MgSO_4$/ 30mM tricine buffer (pH7.8)/ 10mM DTT) in individual wells of a microtiter plate. 100μL of 1 mM D(-)-luciferin (in 0.1M KPO_4 (pH 7.8); Boehringer Mannheim) were added to each well and the amount of light produced measured by using a ML3000 microtiter plate luminometer (under conditions of integrate flash mode, high gain, integrate window = 10 seconds, at a temperature of 22°C). Luciferase activities were calculated as total light output relative to protein content in each cell lysate sample.

The results of these tests are set forth in tabular form below:

TABLE II

For pB-301

	Control	$10^{-9}M$	$10^{-8}M$	$10^{-7}M$	$10^{-6}M$	$10^{-5}M$
Raloxifene	0.7	2.4	9.6	9.63	9.05	5.25
Estradiol	0.7	1.1	3.2	5.5	5.2	8.0
E2 + Ral	---	17.3	16.0	7.3	9.3	10.5

For pGL2ERELUC:

	Control	$10^{-9}M$	$10^{-8}M$	$10^{-7}M$	$10^{-6}M$	$10^{-5}M$
Raloxifene	1.7	1.9	1.5	2.0	1.9	1.9
Estradiol	1.7	5.8	5.5	5.9	6.2	6.5
E2 + Ral	---	1.8	1.9	2.1	2.3	2.4

Representative results of these experiments are shown in Figure 5.

These results clearly demonstrate the existence of a distinct promoter region in the TGFβ gene promoter that is responsive to antiestrogens. While raloxifene acts as a potent antagonist to transcription initiated by ERE-containing promoters of genes such as the vitellogenin gene, it was found herein to act as a super-agonist in inducing transcription from the TGFβ-3 promoter. Interestingly, at low concentrations, raloxifene and estrogen *synergistically* induced transcription from the TGFβ-3 promoter in the reporter plasmids of the invention, suggesting that raloxifene-induced gene transcription may be mediated by a novel mechanism.

EXAMPLE VII

Estrogen Receptor Dependent Gene Activation of TGFβ-3 by Estrogen and Antiestrogens

It was known in the prior art that the ability of both estrogens and antiestrogens to influence TGFβ production is dependent on the expression of estrogen receptor (ER), but the level at which this influence is exerted was not known (i.e. transcriptional, translational or post-translational). A series of experiments were therefore performed to investigate the putative dependence on ER expression of induction of reporter gene expression using the TGFβ promoter-containing constructs of the invention. Lack of ER expression virtually abolishes expression of TGFβ-3, regardless of the presence of estradiol or raloxifene. Through the use of mutant ER proteins, it has been determined that different domains of the ER molecule are responsible for estrogen and raloxifene induction.

A. ER dependent induction of TGFβ-3

Cultures of MG63 were prepared for co-transfection as in Example IV. One such culture was co-transfected with pB2-499 and pRSVER and another was co-transfected with pB2-499 and pRSV vector plasmid (control). The ability of the following compounds to induce transcription via the raloxifene responsive element of the TGFβ-3 gene was then assayed essentially as described in Example V:

(a) ethanol
(b) 17β-estradiol ($10^{-7}M$)
(c) raloxifene ($2 \times 10^{-6}M$)
(d) 17β-estradiol ($10^{-7}M$) and raloxifene ($2 \times 10^{-6}M$)

The results of one such experiment are set forth in the following Table, and a representative example of a thin-layer chromatogram produced thereby is shown in Figure 6.

TABLE III

	Hormone Vehicle	Estradiol	Raloxifene	Estradiol + Raloxifene
Control Plasmid	0.9	1.0	1.1	1.2
ER Expression Plasmid	1.3	4.6	45.7	33.7

These results clearly indicate that ER expression is required for TGFβ-3 gene promoter-mediated induction of reporter gene expression in response to estrogen, antiestrogens and combinations thereof.

B. Analysis of ER protein domains required for induction from the TGFβ-3 promoter

It was disclosed in the prior art that the ER protein region designated "E" is necessary for estrogen binding, while region "C" is necessary for DNA binding. *See* Kumar *et al., EMBO J.*, **9**, 2231-2236 (1986). It has also been well established that the "C" region is essential for ER activation of ERE-containing genes, while the "E" region is required for estrogen-dependent inducibility.

To determine the regions of the ER involved in induction of transcription from the TGFβ-3 promoter, cultures of MG63 cells were prepared for co-transfection as in Example IV. Cells were transfected with mixtures of pTGFβ-301LUC and one of the following expression plasmids comprising various deletion mutants of ER:

a. pCMV-ER
b. pCMV-ERΔA/B
c. pCMV-ERΔB/C/D
d. pCMV-ERΔE/F
e. pCMV-ER_{1-530}
f. pCMV-$ER_{L540}Q$

See Reese & Katzenellenbogen, *J. Biol. Chem.*, **266**, 10880-10887 (1990) and Figure 7 for a further explanation of the extent of each deletion in these plasmids.

The ability of these mutant ERs to mediate raloxifene-induced TGFβ-3 activation was tested by treating co-transfected cells with vehicle (10μL ethanol) or 10^{-7}M raloxifene. The increase of raloxifene-induced luciferase activity over basal activity was calculated as the fold induction by raloxifene in the presence of different mutant ER forms as depicted in Figure 7.

The results of one such experiment are shown in the following Table:

TABLE IV

ER mutant form	vehicle	raloxifene	fold induction
pCMV-ER_{wt}	3.7	32.8	8.9
pCMV-ERΔA/B	14.2	39.4	2.8
pCMV-ERΔB/C/D	21.2	73.4	3.4
pCMV-ERΔE/F	17.4	20.9	1.2
pCMV-ER_{1-530}	13.4	26.5	2.0
pCMV-ER_{L540^Q}	12.2	39.8	3.3

These results show that the hormone binding domain (i.e., the "E" region of the estrogen receptor molecule) is both necessary and sufficient to mediate raloxifene-stimulated, TGFβ-3 promoter-mediated transcription of reporter genes in the reporter plasmids of the invention. The "C" region of the ER molecule appears not to be required for this process. This finding further supports the suggestion that a novel mechanism of activating gene transcription involving ER may be involved in transcription from the TGFβ promoter.

EXAMPLE VIII

Activities of Estrogen and Antiestrogens on TGFβ-3 Promoter

Transcriptional activation of TGFβ promoter-mediated gene expression by estrogen and antiestrogen compounds was found to be concentration-dependent. Cultures of MG63 cells were transiently co-transfected with pB-301 and pRSVER as described in Example V. Such transiently transfected cell cultures were divided into four groups of twelve cultures, and each of the four groups was used to test the ability of one estrogen or antiestrogen compound to induce transcription from the TGFβ-3 promoter individually. For each group of twelve cultures, the particular estrogen or antiestrogen compound was tested in replicate cultures at six concentrations, varying in tenfold increments from 10^{-9}M to 10^{-5}M, as well as one set of replicate cultures treated with vehicle only (for a total of twelve cultures per experimental treatment). Hormones were dissolved in ethanol and applied to the cultures in media as described above. The four estrogens and antiestrogens tested were:

a. 17β-estradiol (Sigma Chemical Corp., St. Louis, MO);
b. raloxifene (Eli Lilly, Indianapolis, IN);
c. tamoxifen (Sigma);
d. ICI 164,384 (described in European Patent No. EP138504, issued 20 July 1988).

After 24 hours of hormone treatment (or control) the cells were washed, harvested, lysed, and assayed for CAT activity as described in Example V. The results of one such experiment are tabulated below and are depicted in Figure 8:

TABLE V

	vehicle	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
estradiol	0.85	0.96	1.1	1.6	4.3	3.4
raloxifene	0.85	6.9	19.2	18.1	19.6	14.8
tamoxifen	0.85	0.91	1.2	0.98	2.9	13.5
ICI 164,384	0.85	0.89	0.88	10.9	15.2	6.5

Although all estrogens and antiestrogens influence TGFβ-3 promoter activity, each compound exhibits its own distinctive dose-response curve. Raloxifene is by far the more potent activator, displaying more than a 20-fold induction of reporter gene transcription and having an ED_{50} at nanomolar concentrations. In contrast, estradiol showed only a 4-fold induction of reporter gene expression and an ED_{50} that was two orders of magnitude higher than that of raloxifene. Tamoxifen activates the TGFβ-3 promoter only at high levels (i.e., greater than micromolar). ICI 164,384 showed an ED_{50} of 10^{-7}M, but this compound appears to be much less active at high concentrations (10^{-5}M). These results demonstrate that a novel element has been found in the promoter region of the TGFβ-3 gene termed a raloxifene responsive element (RRE). This element induces transcription in the presence of both estrogens and antiestrogens and each of these compounds exhibits a characteristic dose-response profile of transcriptional activation.

EXAMPLE IX

Raloxifene-Mediated Transcriptional Activation of the TGFβ-3 Promoter in CHO and MCF-7 Cells

The ability of raloxifene to induce transcription from the TGFβ-3 promoter distinct from estrogen-mediated induction was demonstrated in a variety of cell lines.

A. TGFβ-3 activation in CHO cells

Cultures of CHO cells were transiently co-transfected with pB-301 and pRSVER as described in Example IV and were used to determine the ability of both raloxifene and estradiol to induce transcription via the raloxifene responsive element. Twelve transiently transfected cultures were treated in replicate with either estradiol or raloxifene at six concentrations varying in tenfold increments from 10^{-9}M to 10^{-5}M (as well as a vehicle only control for a total of twelve cultures). Hormones were dissolved in ethanol and applied to the cultures in media as described above.

After 24 hours of incubation with the hormonal preparations (or the control), the cells were washed, harvested, lysed, and assayed for CAT activity as described in Example V. The results are tabulated below and depicted in Figure 9:

TABLE VI

	0M	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
estradiol	10.6	11.9	22.6	29.4	32.9	33.3
raloxifene	10.6	21.3	21.2	19.7	20.8	10.1

These results demonstrated that the previously-observed responsiveness of the TGFβ-3 promoter to estrogen and raloxifene was retained when assayed in CHO cells.

B. TGFβ-3 activation in MCF-7 cells

Cultures of MCF-7 cells were transiently transfected with pTGFβ-301LUC as described in Example IV. These cultures were used to test the ability of raloxifene and estradiol to induce transcription in this cell type. Cultures were treated with either estradiol or raloxifene at one of the following six concentrations: 0M, 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M. Hormones were dissolved in ethanol and applied to the cultures in media as described above.

After 24 hours of treatment with the hormonal preparation (or the vehicle control), the cells were washed, harvested, lysed, and assayed for LUC activity according to the method of Example VI. The results of this experiment are tabulated below, and the results from a series of such experiments are summarized in Figure 10:

TABLE VII

	0M	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
estradiol	7.8	19.2	9.1	107.3	101.3	122
raloxifene	7.8	158	309	314	451	206

(Luciferase activity expressed in thousands of units)

Similar assays were performed in human endometrial cancer cells (RL59.2), human cervical cancer cells (HeLa), and monkey kidney cells (COS-1) (American Type Culture Collection, Rockville, MD). Transcription initiated by the TGFβ-3 promoter was found to be induced by estrogen and raloxifene in all cell types tested (with variations in the magnitude of induction). These results demonstrate that estrogen and raloxifene-mediated induction of reporter gene transcription from the TGFβ-3 promoter is not restricted to specific cell types. The different levels of induction in different cells, however, might indicate the abundance of other factors in these cells involved in regulation. The fact that raloxifene and estrogen responsiveness of the TGFβ-3 promoter were found using both luciferase and CAT as reporter genes indicates that this regulation is a general characteristic of gene expression from this promoter.

EXAMPLE X

Comparative Induction of Reporter Gene Expression from the TGFβ-3 Promoter by Raloxifene and Related Compounds

Antiestrogen compounds were known in the prior art to be capable of inducing TGFβ gene expression in a dose-dependent manner. Knabbe *et al., Am. J. Clin. Onc.*, **14** (Suppl.2), S15-S20 (1991). Furthermore, as shown in Example VIII above, raloxifene and tamoxifen were found to be capable of inducing TGFβ-3 gene expression in a dose dependent manner.

The experiments described in this Example were performed in order to correlate the ability of compounds to induce transcription via the raloxifene responsive element of the TGFβ-3 promoter with their known uterotropic capacities as demonstrated in ovariectomized rats. Seven compounds that are structurally related to raloxifene were tested for their ability to induce transcription via the raloxifene responsive element of the TGFβ-

3 promoter. These compounds can be distinguished on the basis of a spectrum of in vivo activity ranging from uterotropic (LY112676, LY81099, and LY13314) to anti-uterotropic (LY113526, LY139482 and LY177366), and included a compound known to be inert *in vivo* (LY98005).

The IUPAC names for the compounds and the U.S. Patents in which they have been claimed are as follows:

113526	2-(p-hydroxyphenyl)benzo[B]thien-3-YL p-[2-(1-pyrrolidinyl)ethoxy] phenyl ketone	4,133,814
139482	[4-[2-(hexahydro-1H-azepin-1-yl) ethoxy]phenyl][6-hydroxy-2-(4-hydroxyphenyl)benzo[B]thien-3-yl] methanone	4,380,635
177366	[6-(2,2-dimethyl-1-oxopropoxy)-2-[4-(2,2-dimethyl-1-oxopropoxy) phenyl]benzo[B]thien-3-yl][4-[2-1-piperidinyl)ethoxy]phenyl] methanone hydrochloride	07/902,933, filed July 28, 1992, incorporated by reference)
98005	p-hydroxyphenyl 3-(-hydroxyphenyl)benzo[B]thien-2-yl ketone	
112676	(p-hydroxyphenyl) 5-hydroxy-3-phenylbenzo[B] thien-2-yl ketone	4,075,227
81099	p-hydroxyphenyl 3-phenylbenzo[B] thien-2-yl ketone	4,075,227
133314	[3,4-dihydro-2-(4-methoxyphenyl)-1-naphthanlenyl] [4-[2-(1-pyrrolidinyl)ethoxy]phenyl] methanone, methanesulfonic acid salt	

These compounds are depicted in Figure 11.

Raloxifene, LY81099, LY98005, LY112676, LY113526, LY13314, LY139482, and LY177366 (Eli Lilly and Company, Indianapolis, Indiana) were assayed to compare their ability to induce transcription from the promoter of the TGFβ-3 gene at varying concentrations. Cultures of MG63 cells transiently co-transfected with pB-301 and pRSVER were treated as in Example VI with the above compounds at concentrations of 0M, 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M. The experimental results are shown in tabular form and depicted in Figure 12.

TABLE VIII

	0M	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
raloxifene	0.61	2.1	6.4	7.5	7.0	4.0
LY81099	0.61	0.6	0.5	0.7	0.9	2.3
LY98005	0.61	0.4	0.5	0.5	0.4	0.5
LY112676	0.61	0.6	0.6	0.5	1.7	3.8
LY113526	0.61	0.7	0.7	0.6	2.0	2.1
LY133314	0.61	0.7	0.7	3.1	6.6	1.0
LY139482	0.61	0.8	2.5	2.7	2.5	2.0
LY177366	0.61	1.1	7.9	10.2	7.9	6.1

Compounds that displayed uterotropic properties in *vivo* (i.e., estrogenic compounds) showed ED_{50} values of approximately 10^{-7}M and had a relatively lower-fold induction of reporter gene expression from the TGFβ-3 promoter, much like the profile exhibited by estrogen in Example VIII. In contrast, compounds that have a profile similar to raloxifene *in vivo* demonstrate an ED_{50} of 10^{-9}M and a relatively greater fold induction than estrogenic compounds. *In vivo* data regarding raloxifene was set forth in U.S. Patent Appln. Ser. No. 07/920,933, filed July 28, 1992, and is incorporated by reference. In summary, compounds exerting estrogen like qualities *in vivo* show significantly less induction of transcription via the TGFβ-3 promoter than compounds that exerted antiestrogen-like qualities *in vivo.*

The results of these experiments demonstrate the utility of the reporter gene-containing expression plasmids described herein as a screening technique for identifying potential anti-osteoporosis agents, because those compounds that are raloxifene-like in their induction profiles and ED_{50}'s show relatively lower uterotropic effects than estrogen-like compounds having lower induction profiles and higher ED_{50}'s in the present assay.

EXAMPLE XI

Localization of the Raloxifene Response Element in TGFβ-3 Promoter in the Region from +35 to +75

At least a portion of the raloxifene response element (RRE) in the human TGFβ-3 gene promoter was approximately localized to a particular 41 nucleotide sequence found at positions +35 through +75. This sequence was found to be necessary for mediating raloxifene-induced transcriptional activation of reporter gene expression in the TGFβ-reporter gene expression constructs described above.

A. Identification of a raloxifene responsive element by functional analysis of TGFβ-3 promoter deletion mutants prepared in vitro

Cultures of MG63 cells transiently co-transfected with pCMVER and one of a variety of TGFβ-3 promoter deletion reporter constructs (including pB-499, pB-301, pB-221, pB-91, pB-60, pB-47, pTGFβ-38LUC, pTGFβ+75LUC, pTGFβ+35LUC and pLUC) were generated as described in Example I. These cultures were then treated with either ethanol (as a control) or 10^{-6}M raloxifene. The degree of induction of reporter gene expression after treatment with raloxifene relative to that obtained by treatment with vehicle alone was calculated for each TGFβ-3 promoter deletion construct and are tabulated below and are depicted in Figure 13:

TABLE IX

Vector Plasmid	TGFβ-3 Promoter region	Fold induction by raloxifene
pB-499	-499 - +110	6.8
pB-301	-301 - +110	13.1
pB-221	-221 - +110	8.7
pB-91	-91 - +110	10.1
pB-60	-60 - +110	12.5
pB-47	-47 - +110	11.5
TGFβ-38LUC	-38 - +110	7.1
TGFβ+75LUC	-38 - +75	5.8
TGFβ+35LUC	-38 - +35	1.2
pLUC vector alone		0.5

These results localize at least one portion of the raloxifene responsive element to positions +35 to +75 in the TGFβ-3 promoter sequence.

B. Nucleotide sequence of the raloxifene responsive element

The nucleotide sequence of the TGFβ-3 promoter from position -38 to +110 was depicted in Figure 14. The raloxifene responsive sequence was found above to be the sequence depicted in the Figure in outline form. The open arrow indicates the major transcription start site (+1). The two black arrows indicate the two minor transcription start sites. The "TATA" sequence is shown in the open box. A putative CCCTC-motif is indicated by a series of horizontal arrow heads under the sequence of the putative raloxifene responsive element. *See* Lobanenekov *et al. Oncogene*, **5**, 1743-1753 (1990).

Two conclusions can be drawn from the TGFβ-3 analysis. The first is that no palindromic sequences homologous to the ERE was found in this region of the TGFβ-3 promoter. This finding is consistent with the results shown in Example VII which demonstrated that DNA binding activity of ER is not required. Second, ER-mediated raloxifene activation of TGFβ-3 most likely requires other factors that are capable of binding to the raloxifene responsive sequence. A good candidate for such a protein is the CTCF factor identified by Lobanenkov *et al.* which is involved in *c-myc* gene regulation. These findings may lead to the identification of other genes as potential raloxifene inducible genes that have raloxifene responsive elements in their promoters. Furthermore, such genes could be used to identify genetic elements having the activity of raloxifene responsive elements for use in the screening procedure set forth in Example XIII.

The raloxifene responsive element of the present invention was used to search the GenBank sequence library; significant homology was found between this element and elements in the following genes:

GenBank/EMBL Data Bank	
Accession No.:	Gene:
X56595	Chicken type VI collagen _-2
X55373	Human ETS-2 promoter region
M30137	Human ETS-2 (5' flank)
D10231	Mouse glucose transporter (enhancer 2)
M12731	Mouse N-myc proto-oncogene
M13945	Mouse pim-1 proto-oncogene
X63281	*R. norvegicus* N-myc gene
X16995	Mouse N10 gene
M94152	Rat adenosine receptor
M20131	Rat cytochrome P450IIE1
M34111	Rat PTHrP
J05097	Rat substance P receptor
M64236	Rat substance P receptor

This finding supports the existence of this element as a discrete and important regulatory unit capable of mediating pleiotropic physiological effects *in vivo* in a variety of tissues and cell types.

EXAMPLE XII

Estrogen and Raloxifene Induce LDL Receptor Promoter Activation

LDL receptor expression plays an essential role in regulation of serum LDL-cholesterol uptake. It has been known that estrogen induces LDL receptor messenger RNA *in vivo*. Ma *et al., Proc. Natl. Acad. Sci. USA*, **83**, 792-796 (1986). As shown in this Example, this activation of LDL receptor promoter sequence by estrogen is mediated by ER. Raloxifene also induces LDL receptor promoter; however, this induction is ER independent.

A. Estrogen and antiestrogen induced LDLR-Luc production in presence of ER

ATCC strain HepG2 cells were co-transfected with pLDLRLUC10 and pRSVER as described in Example IV. These cells were exposed to estradiol and raloxifene under the conditions set forth in Example VI. The results are tabulated below and a series of experiments are depicted in Figure 15:

TABLE X

	0M	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
estradiol	20.9	16.6	15.4	40.7	74.0	69.0
raloxifene	20.9	20.9	19.9	26.9	14.6	14.6

B. Estrogen and antiestrogen-induced LDLR-Luc production in the Absence of ER

ATCC strain HepG2 cells were co-transfected with pLDLRLUC10 and pRSV vector plasmid as described in Example IV. These cells were exposed to estradiol and raloxifene under the conditions set forth in Example VI. The results are tabulated below and a representative series of such experiments are set forth in Figure 16:

TABLE XI

	0M	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
estradiol	1597	1645	1792	1574	1578	2445
raloxifene	1597	1652	1234	1025	1291	5561

These results show that both raloxifene and estrogen have the ability to induce LDL receptor expression. This result provides an explanation of serum lipid lowering effect by estrogen and raloxifene *in vivo* in both animal models and humans.

EXAMPLE XIII

Method for Screening Potential Anti-Osteoporosis Agents

Based on the foregoing Examples, an assay using luciferase as a reporter gene was designed to screen for potential anti-osteoporosis agents.

Cultures of MCF-7 cells were stably transfected with:

1. pTGFβ-301LUC;
2. pGL2LUC; or
3. pGL2ERELUC;

using the methods described in Example IV. The cells are then used in the inventive method depicted in Figure 17.

STEP 1. The first procedure that is used in the screening assay is to determine the ability of a compound to induce transcription from the TGFβ-3 promoter. The assay is performed essentially as described in Example IX, using MCF-7 cells stably transfected with pTGFβ-301LUC. Cell culture and assay conditions are adapted to the 96-well microtiter plate format. Cells are seeded in a 96-well plate at a density resulting in approximately 50% confluency. Test compounds may be selected from a variety of sources, including pharmaceutical research records, chemical manufacturers products lists, and naturally-occurring sources such as fermentation extracts. Cells are incubated in growth media (as described in Example IV) containing the test compound for about 24 hours. Cells are then lysed *in situ* on the plate, and the lysates subjected to both a quantitative protein assay and to the luciferase activity assay. Compounds that induce a greater than two-fold increase in luciferase activity are considered competent for further testing.

STEP 2. Assays are performed with compounds identified as described in Step 1 on cell cultures that have been stably transfected with pGL2LUC to determine whether such compounds are general transcription inducers. As such general transcriptional inducers lack the transcriptional induction specificity required for potential anti-osteoporetics that are modulators of raloxifene-responsive element-dependent gene expression, such general inducers are excluded from further testing.

STEP 3. Compounds having the required transcriptional induction specificity (that is, are capable of inducing transcription induction in pTGFβ-301LUC cells without inducing transcription in cells transfected with pGL2LUC) for potential anti-osteoporetics, that are modulators of raloxifene-responsive element-dependent gene expression, are then assayed to determine whether such compounds induce transcription from an estrogen responsive element. Cells stably transfected with pGL2ERELUC are assayed as described in Example VI both in the presence and in the absence of estradiol. Compounds that activate pGL2ERELUC in the *absence* of estrogen are disqualified for further testing, because the capacity of these compounds to induce transcription from an estrogen-responsive element in the absence of estrogen evidences potential estrogenic activity *in vivo*.

STEP 4. Compounds that have fulfilled the criteria of Steps 1 through 3 are then further tested to determine whether such compounds are either anti-estrogenic or non-estrogenic/non-antiestrogenic. To this end, the compounds are assayed in the presence of estradiol in cells stably transfected with pGL2ERELUC. Inhibition of estrogen-induced luciferase activity in this assay indicates that such compounds have anti-estrogenic activity. Both anti-estrogenic and non-estrogenic compounds will be characterized for their dose-response profiles and ED_{50} values. Further experiments may be done to establish the dose-response profiles of such compounds and to compare them with known anti-estrogens like raloxifene. *See* Example X.

Following this screening protocol, conventional assays, particularly an *in vivo* assay involving appropriate animal model systems, may be used to further characterize the anti-estrogenic properties of the compounds identified as described herein. Development of such anti-estrogenic compounds having desirable anti-

osteoporotic properties may then be advantageously and expeditiously achieved from the compounds identified in this assay.

EXAMPLE XIV

Correlation between *in vitro* and *in vivo* activity

The following experiments were performed to demonstrate the correlation between the *in vitro* assay and an *in vivo* model of post-menopausal osteoporosis. The *in vitro* assay measures the test compound's ability to induce transcription via the raloxifene responsive element of the TGFβ-3 promoter. The *in vivo* model measures the changes in bone (femur) mineral density in ovariectomized rats. The following compounds were used in these experiments:

raloxifene	6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-piperidinoethoxy)benzoyl]benzo[b]thiophene
088074	6-hydroxy-2-(4-hydroxyphenyl)-3-(4-hydroxybenzoyl)benzo[b]thiophene
156678	6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-(3-methylpyrrolidine)ethoxyl)benzoyl]benzo[b]thiophene
171147	2-(4-hydroxyphenyl)-3-[4-(2-piperidinoethoxy)benzoyl]benzo[b]thiophene
309503	6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-hydroxyethoxy)benzoyl]benzo[b]thiophene

These compounds are prepared as generally described in U.S. Patent No. 4,133,814 (issued January 9, 1979) which is incorporated herein by reference.

Raloxifene, 088074, 156678, 171147, and 309503 (Eli Lilly and Company) were assayed to determine their ability to produce a two-fold induction in the transcription from the promoter of the TGFβ-3 gene, as measured by luciferase activity, at a concentration of less than 10 nM [i.e. minimum effective concentration (MEC) < 10 nM]. One day prior to transfection, HeLa cells were trypsinized with a solution of 0.05% trypsin/5.3mM tetrasodium ethylenediamine tetraacetate (EDTA) to achieve a single cell suspension. The detached confluent cells were counted and diluted to a concentration of 1,000,000 cells/mL. Three million cells and 3:1 media (25mL) were added to t-150 flasks and incubated overnight. The next day, the media was removed from the cells and replaced with fresh media (25mL). The cells were transiently co-transfected with pTGFβ-301LUC (10μg), pCMVER (1μg), and pRSVβgal (1μg) using the ProFection Mammalian Transfection System (Promega). A transfection solution was prepared by mixing pTGFβ-301LUC (940μg), pCMVER (94μg), pRSVβgal (94μg), $CaCl_2$ (5.704mL), and nuclease free water (47ml) with 2XHEPES (47ml) while vortexing. The resulting solution was incubated at room temperature for 30 minutes. This transfection solution (3mL) was added to each flask.

After incubating the cells with the transfection solution overnight, the media was removed and each flask washed with Ca/Mg free phosphate buffered saline solution (10mL, GIBCO). The washed cells were trypsinized (3mL trypsin/EDTA) to detach the cells. The detached cells were treated with fresh media (3mL). A pellet of the cells was prepared by centrifuging and removing the media and trypsin. The cells were resuspended in approximately 20mL of media and counted. The cells were diluted to a concentration of 500,000 cells/mL. Next, 50,000 cells (100μL) were added to each well of a 96-well plate, and the cells incubated overnight.

The test compounds were dissolved in dimethyl sulfoxide and diluted initially to 1:1000. This dilution was accomplished by adding 2μL of drug solution to 1000μL of media in a deep well microtiter plate (1:500 dilution), then 100μL of the dilute drug solution is added to 100μL of media already in the plate (1:2 dilution). For compound screening, the compounds are diluted 1:1000, as previously described, then further diluted 1:100. Alternatively, the minimum effective concentration is determined using an eight-dilution dose-response curve. The compounds and cells are incubated in 96-well plates overnight.

The media was removed from the cells and each well washed with Ca/Mg free phosphate buffered saline (200μL). The saline solution was removed and the cells lysed with 60μL of lysis buffer (100mM KPO_4, 0.2% Triton X-100, 1mM DTT, pH 7.8). The resulting solution was used to assay for luciferase or β-gal activity. The luciferase assay was performed substantially as described in Example VI, except the luciferin solution comprises 100mg luciferin, 9mL 1M Glycyl-gylcine, 36mL 40mM EGTA, 720mL 1M DTT, 5.4mL 1M $MgSO_4$, and 310mL H_2O. The results of these experiments are shown in tabular form in Table XII.

TABLE XII

compound	TGFβ assay[a]	bone density[b]
raloxifene	+	+
088074	-	-
156678	+	+
171147	+	+
309503	-	-

a "+" indicates the test compound produced a two-fold induction in normalized luciferase activity with an ED_{50} < 10nM

b "+" indicates the bone mineral density is statistically higher than ovariectomized animals

The above compounds were also tested for their ability to preserve bone mineral density in ovariectomized rats. Seventy-five day old female Sprague Dawley rats (weight range of 225 to 275 g) were obtained from Charles River Laboratories (Portage, MI). They were housed in groups of three and had *ad libitum* access to food (calcium context approximately 1%) and water. Room temperature was maintained at 22.2°C ± 1.7°C with a minimum relative humidity of 40%. The photoperiod in the room was 12 hours light and 12 hours dark.

One week after arrival, the rats underwent bilateral ovariectomy under anesthesia [44 mg/kg Ketamine and 5 mg/kg Xylazine (Butler, Indianapolis, IN) administered intramuscularly]. Treatment with vehicle, or a test compound was initiated on the day of surgery following recovery from anesthesia. Oral dosage was by gavage in 0.5 mL of 1% carboxymethylcellulose (CMC). Body weight was determined at the time of surgery and weekly thereafter and the dosage was adjusted with changes in body weight. Vehicle or treated ovariectomized (ovex) rats and non-ovariectomized (intact) rats were evaluated in parallel with each experimental group to serve as negative and positive controls.

The rats were treated daily for 35 days (6 rats per treatment group) and sacrificed by decapitation on the 36th day. The 35 day time period was sufficient to allow maximal reduction in bone density, measured as described herein. The right femurs were excised and scanned at the distal metaphysis 1 mm from the patellar groove with single photon absorptiometry. Results of the densitometer measurements represent a calculation of bone density as a function of the bone mineral context and bone width. Generally, ovariectomy of the rats caused a reduction in femur density of about 25% as compared to intact vehicle treated controls. The results of these experiments are shown in Table XII.

The results of these experiments show that the compounds that potently induce transcription from the raloxifene responsive element of promoter region of the TBFβ-3 gene, such as raloxifene, 156678, and 171147, also show preservation of bone density in ovariectomized rats.

The compounds that fail to induce transcription for the raloxifene responsive element, such as 088074 and 309503, also fail to show a statistically significant protection against bone loss in mineral density over ovariectomized rats.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit or scope of the invention as set forth in the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ELI LILLY AND COMPANY
(B) STREET: Lilly Corporate Center
(C) CITY: Indianapolis
(D) STATE: Indiana
(E) COUNTRY: United States of America
(F) ZIP: 46285

(ii) TITLE OF INVENTION: MATERIALS AND METHODS FOR SCREENING ANTI-OSTEOPOROSIS

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
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(D) STATE: Surrey
(E) COUNTRY: United Kingdom
(F) ZIP: GU20 6PH

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Macintosh
(C) OPERATING SYSTEM: Macintosh 7.0
(D) SOFTWARE: Microsoft Word 5.1

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2205 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..2
(D) OTHER INFORMATION: /note= "Number 1 corresponds to -1362 of TGFB-1 promoter"

(ix) FEATURE:
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(B) LOCATION: 1363..1365
(D) OTHER INFORMATION: /note= "Corresponds to +1 codon of TGFB-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGAGAGGAAA AGACTGGGCC TGGGGTCTCC AGTGAGTATC AGGGAGTGGG GAATCAGCAG    180
GAGTCTGGTC CCCACCCATC CCTCCTTTCC CCTCTCTCTC CTTTCCTGCA GGCTGGCCCC    240
GGCTCCATTT CCAGGTGTGG TCCCAGGACA GCTTTGGCCG CTGCCAGCTT GCAGGCTATG    300
GATTTTGCCA TGTGCCCAGT AGCCCGGGCA CCCACCAGCT GGCCTGCCCC ACGTGGCGGC    360
CCCTGGGCAG TTGGCGAGAA CAGTTGGCAC GGGCTTTCGT GGGTGGTGGG CCGCAGCTGC    420
TGCATGGGGA CACCATCTAC AGTGGGGCCG ACCGCTATCG CCTGCACACA GCTGCTGGTG    480
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CCCCATGTTG ACAGACCCTC CTTCTCCTAC CTTGTTTCCC AGCCTGACTC TCCTTCCGTT    960
CTGGGTCCCC CTCCTCTGGT CGGCTCCCCT GTGTCTCATC CCCCGGATTA AGCCTTCTCC   1020
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CCCGCTTTGG TGGCGCTTGG CCGCCAGGAG GCAGCACCCT GTTTGCGGGG CGGAGCCGGG   1140
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GGAGACGGAT CTCTCTCCGA CCTGCCACAG ATCCCCTATT CAAGACCACC CACCTTCTGG    1980
TACCAGATCG CGCCCATCTA GGTTATTTCC GTGGGATACT GAGACACCCC CGGTCCAAGC    2040
CTCCCCTCCA CCACTGCGCC CTTCTCCCTG AGGAGCCTCA GCTTTCCCTC GAGGCCCTCC    2100
TACCTTTTGC CGGGAGACCC CCAGCCCCTG CAGGGGCGGG GCCTCCCCAC CACACCAGCC    2160
CTGTTCGCGC TCTCGGCAGT GCCGGGGGGC GCCGCCTCCC CCATG                    2205
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(2) INFORMATION FOR SEQ ID NO:2:

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          (A) LENGTH: 5578 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
          (A) NAME/KEY: TATA_signal
          (B) LOCATION: 2248..2252

    (ix) FEATURE:
          (A) NAME/KEY: exon
          (B) LOCATION: 2278..3980
    (ix) FEATURE:
          (A) NAME/KEY: intron
          (B) LOCATION: 3981..5578
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(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 3635..3980
(D) OTHER INFORMATION: /note= "CDS, Codon start = 1"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..2
(D) OTHER INFORMATION: /note= "Number 1 corresponds to TGFB-2 -2277"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 3303 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
   (A) NAME/KEY: mRNA
   (B) LOCATION: 2170..3303

(ix) FEATURE:
   (A) NAME/KEY: mRNA
   (B) LOCATION: 2214..3303

(ix) FEATURE:
   (A) NAME/KEY: mRNA
   (B) LOCATION: 2219..3303

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(ix) FEATURE:
       (A) NAME/KEY: misc_RNA
       (B) LOCATION: 3301..3303
       (D) OTHER INFORMATION: /note= "CDS Start, codon start = 1,
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(ix) FEATURE:
       (A) NAME/KEY: TATA_signal
       (B) LOCATION: 2170..2176

(ix) FEATURE:
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       (D) OTHER INFORMATION: /note= "pB-301   -301 to +110"

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       (B) LOCATION: 1976..2306
       (D) OTHER INFORMATION: /note= "pB-221   -221 to +110"

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       (D) OTHER INFORMATION: /note= "pB-91  -91 to +110"

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       (D) OTHER INFORMATION: /note= "pB-47   -47 to +110"

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       (D) OTHER INFORMATION: /note= "pB-38   -38 to +110"

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       (D) OTHER INFORMATION: /note= "TGFB-3 position  -38 to
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       (D) OTHER INFORMATION: /note= "TGFB-3 position  -38 to
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ATG 3303

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGAGTTTGA TCAGGTCACT GTGACCTGAC TTTTC 35

Claims

1. A nucleic acid comprising a raloxifene responsive element isolated from a promoter region of a TGFβ gene wherein the human TGFβ gene is TGFβ-2 or TGFβ-3.

2. A nucleic acid according to Claim 1 wherein the nucleotide sequence of the nucleic acid comprises a sequence selected from the group consisting of sequences corresponding to 1896 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 1976 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2106 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2137 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2150 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2159 to 2306 shown in Figure 3 and SEQ ID NO:3, sequences corresponding to 2159 to 2271 shown in Figure 3 and SEQ ID NO:3, and sequences

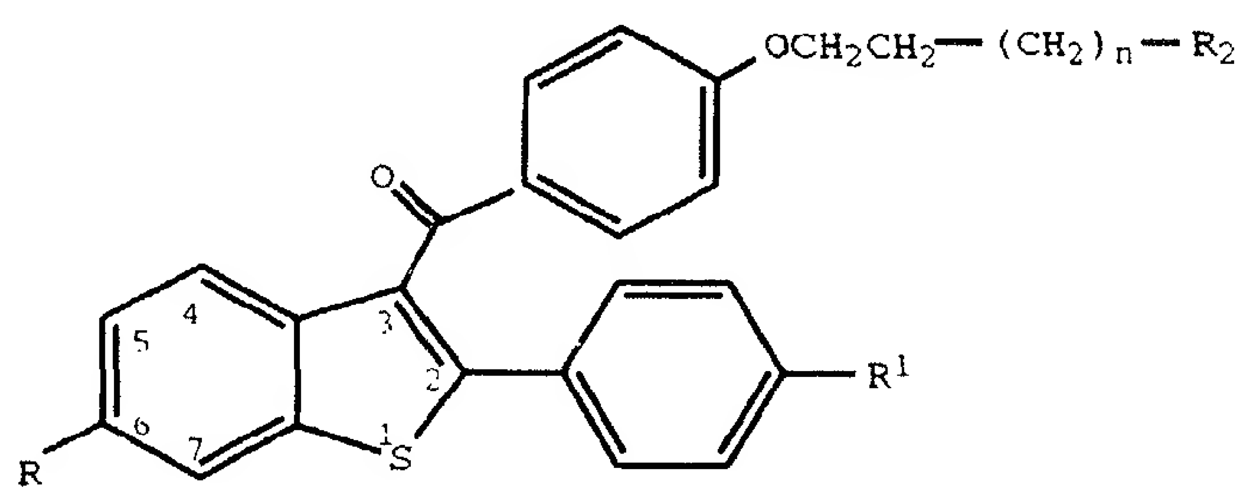
corresponding to 2159 to 2231 shown in Figure 3 and SEQ ID NO:3.

3. A recombinant expression construct comprising the nucleic acid according to Claim 1 or Claim 2 and a reporter gene.

4. A eukaryotic cell transfected with the recombinant expression construct according to Claim 3.

5. A method for screening a multiplicity of compounds to identify compounds having potential as anti-osteoporosis agents, the method comprising identifying a compound of the multiplicity that is capable of inducing transcription from a raloxifene responsive element of a mammalian promoter, that is not a non-specific transcription inducer, is not capable of inducing transcription from an estrogen-responsive element of a mammalian promoter and that is an anti-estrogenic or non-estrogenic compound, the method comprising the steps of:
(a) assaying for the ability of the compound to induce transcription from a raloxifene responsive element of the mammalian promoter;
(b) assaying for the inability of the compound to induce transcription from a mammalian promoter not having a raloxifene responsive element;
(c) assaying for the inability of the compound to induce transcription from an estrogen responsive promoter; and
(d) assaying for the ability of the compound to inhibit estrogen induction of transcription from an estrogen responsive promoter in the presence of estrogen.

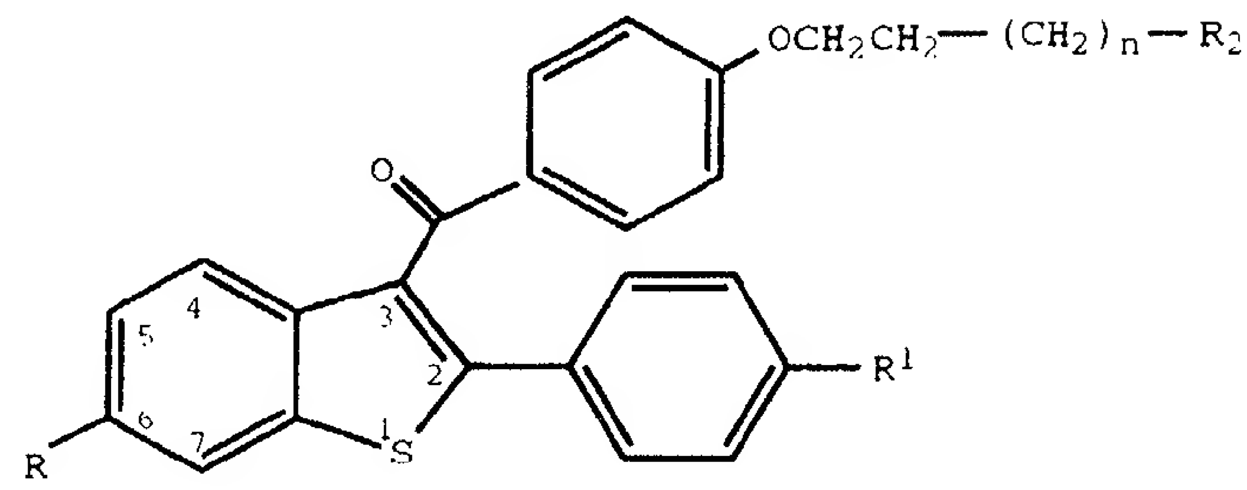
6. A method for inducing bone formation in a mammal which comprises administering a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene, provided the compound is other than a compound of the formula



(I)

wherein
n is 0, 1 or 2;
R and R^1, independently, are hydrogen, hydroxyl, C_1-C_6-alkoxy, C_1-C_6-acyloxy, C_1-C_6-alkoxy-C_2-C_6-acyloxy, R^3-substituted aryloxy, R^3-substituted aroyloxy, R^4-substituted carbonyloxy, chloro, or bromo;
R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;
R^3 is C_1-C_3-alkyl, C_1-C_3-alkoxy, hydrogen, or halo; and
R^4 is C_1-C_6-alkoxy or aryloxy; or
a pharmaceutically acceptable salt thereof.

7. A method for treating osteoporosis which comprises administering a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene, provided the compound is other than a compound of the formula



(I)

wherein

n is 0, 1 or 2;

R and R^1, independently, are hydrogen, hydroxyl, C_1-C_6-alkoxy, C_1-C_6-acyloxy, C_1-C_6-alkoxy-C_2-C_6-acyloxy, R^3-substituted aryloxy, R^3-substituted aroyloxy, R^4-substituted carbonyloxy, chloro, or bromo;

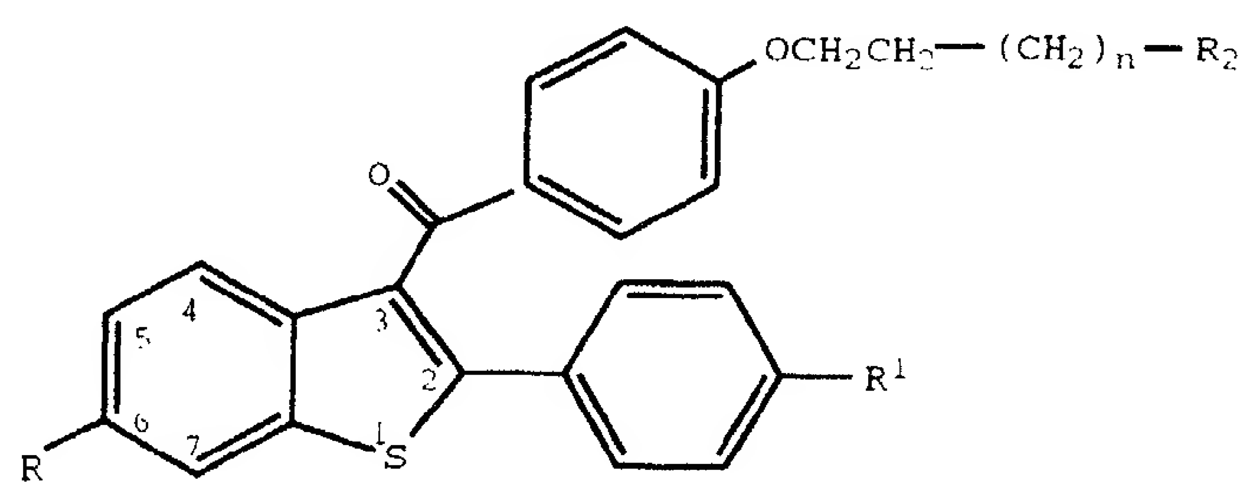
R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;

R^3 is C_1-C_3-alkyl, C_1-C_3-alkoxy, hydrogen, or halo; and

R^4 is C_1-C_6-alkoxy or aryloxy; or

a pharmaceutically acceptable salt thereof.

8. A method for treating bone fractures which comprises administering a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene, provided the compound is other than a compound of the formula



(I)

wherein

n is 0, 1 or 2;

R and R^1, independently, are hydrogen, hydroxyl, C_1-C_6-alkoxy, C_1-C_6-acyloxy, C_1-C_6-alkoxy-C_2-C_6-acyloxy, R^3-substituted aryloxy, R^3-substituted aroyloxy, R^4-substituted carbonyloxy, chloro, or bromo;

R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;

R^3 is C_1-C_3-alkyl, C_1-C_3-alkoxy, hydrogen, or halo; and

R^4 is C_1-C_6-alkoxy or aryloxy; or

a pharmaceutically acceptable salt thereof.

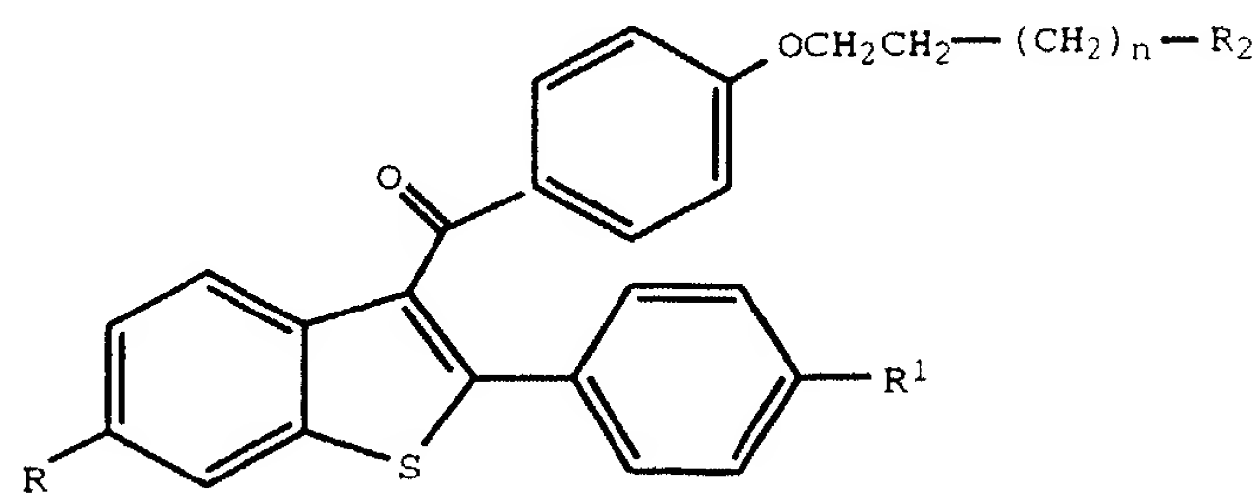
9. A method for inducing bone formation in a mammal which comprises administering a compound that:

(a) potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene;

(b) does not induce transcription from a mammalian promoter not having a raloxifene responsive element;

(c) does not induce transcription from an estrogen responsive promoter; and
(d) inhibits estrogen-induced transcription from an estrogen responsive promoter in the presence of estrogen;

provided the compound is other than a compound of the formula



(I)

wherein

n is 0, 1 or 2;

R and R^1, independently, are hydrogen, hydroxyl, C_1-C_6-alkoxy, C_1-C_6-acyloxy, C_1-C_6-alkoxy-C_2-C_6-acyloxy, R^3-substituted aryloxy, R^3-substituted aroyloxy, R^4-substituted carbonyloxy, chloro, or bromo;

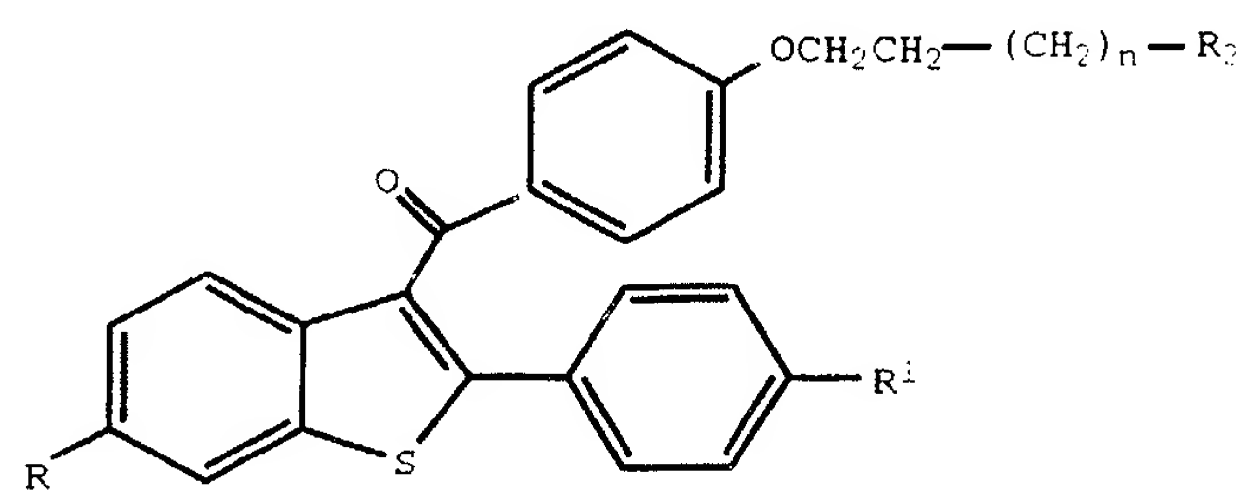
R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;

R^3 is C_1-C_3-alkyl, C_1-C_3-alkoxy, hydrogen, or halo; and

R^4 is C_1-C_6-alkoxy or aryloxy; or

a pharmaceutically acceptable salt thereof.

10. The use of a compound that, when bound to an estrogen receptor, potently induces transcription from a raloxifene responsive element of a promoter region of a TGFβ-3 gene, provided the compound is other than a compound of the formula



(I)

wherein

n is 0, 1 or 2;

R and R^1, independently, are hydrogen, hydroxyl, C_1-C_6-alkoxy, C_1-C_6-acyloxy, C_1-C_6-alkoxy-C_2-C_6-acyloxy, R^3-substituted aryloxy, R^3-substituted aroyloxy, R^4-substituted carbonyloxy, chloro, or bromo;

R^2 is a heterocyclic ring selected from the group consisting of pyrrolidino, piperidino, or hexamethyleneimino;

R^3 is C_1-C_3-alkyl, C_1-C_3-alkoxy, hydrogen, or halo; and

R^4 is C_1-C_6-alkoxy or aryloxy; or

a pharmaceutically acceptable salt thereof; in the preparation of a medicament useful for treating osteoporosis.

FIG. 1A

-1362 GGATCCTTAGCAGGGGAGTAACATGGATTTGGAAAGATCACTTTGGCTGCTGTGTGGGGATAGATAAGACGGTGGGAGCCTAGAAAGGAGGCTGGGTTGG

-1262 AAACTCTGGACAGAAACCCAGAGAGGAAAAGACTGGGCCTGGGGTCTCCAGTGAGTATCAGGGAGTGGGGAATCAGCAGGAGTCTGGTCC[illegible]CACCCATC

-1162 CCTCCTTTCCCCTCTCTCTCCTTTCCTGCAGGCTGGCCCCGGCTCCATTTCCAGGTGTGGTCCCAGGACAGCTTTGGCCGCTGCCAGCTTGCAGGCTATG

-1062 GATTTTGCCATGTGCCCAGTAGCCCGGGCACCCACCAGCTGGCCTGCCCCACGTGGCGGCCCCTGGGCAGTTGGCGAGAACAGTTGGCACGGGCTTTCGT

-962 GGGTGGTGGGCCGCAGCTGCTGCATGGGGACACCATCTACAGTGGGGCCGACCGCTATCGCCTGCACACAGCTGCTGGTGGCACCGTGCACCTGGAGATC

-862 GGCCTGCTGCTCCGCAACTTCGACCGCTACGGCGTGGAGTGCTGAGGGACTCTGCCTCCAACGTCACCACCATCCACACCCCGGACACCCAGTGATGGGG

-762 GAGGATGGCACAGTGGTCAAGAGCACAGACTCTAGAGACTGTCAGAGCTGACCCCAGCTAAGGCATGGCACCGCTTCTGTCCTTTCTAGGACCTCGGGGT

-662 CCCTCTGGGCCCAGTTTCCCTATCTGTAAATTGGGGACAGTAAATGTATGGGGTCGCAGGGTGTTGAGTGACAGGAGGCTGCTTAGCCACATGGGAGGTG

-562 CTCAGTAAAGGAGAGCAATTCTTACAGGTGTCTGCCTCCTGACCCTTCCATCCCTCAGGTGTCCTGTTGCCCCCTCCTCCCACTGACACCCTCCGGAGGC

FIG. 1B

-462 CCCCATGTTGACAGACCCTCCTTCTCCTACCTTGTTTCCCAGCCTGACTCTCCTTCCGTTCTGGTCCCCCTCCTCTGGTCGGCTCCCCTGTGTCTCATC

-362 CCCCGGATTAAGCCTTCTCCGCCTGGTCCTCTTTCTCTGGTGACCCACACCGCCCGCAAAGCCACAGCGCATCTGGATCACCCGCTTTGGTGGCGCTTGG

-262 CCGCCAGGAGGCAGCACCCTGTTTGCGGGGCGGAGCCGGGGAGCCCGCCCCTTTCCCCCAGGGCTGAAGGGACCCCCCTCGGAGCCCGCCCACGCGAGA

-162 TGAGGACGGTGGCCCAGCCCCCCCATGCCCTCCCCCTGGGGCCCGCCCCGCTCCCGCCCCGTGCGCTTCCTGGGTGGGGCCGGGGGCGGCTTCAAAACC

+1

-1

-62 CCCTGCCGACCCAGCCGGTCCCCGCCGCCGCCGCCCTTCGCGCCCTGGGCCATCTCCCTCCCACCTCCCTCCGCGGAGCAGCCAGACAGCGAGGGCCCCG

39 GCCGGGGGCAGGGGGACGCCCCGTCCGGGGCACCCCCCCGGCTCTGAGCCGCCCGCGGGCCGGCCTCGGCCCGGAGCGGAGGAAGGAGTCGCCGAGGA

139 GCAGCCTGAGGCCCCAGAGTCTGAGACGAGCCGCCGCCGCCCCGCCACTGCGGGGAGGAGGGGGAGGAGGAGCGGGAGGAGGGACGAGCTGGTCGGGAG

+271

239 AAGAGGAAAAAAACTTTTGAGACTTTTCCGTTGCCGCTGGGAGCCGGAGGCGCGGGGACCTCTTGGCGCGACGCTGCCCCGCGAGGAGGCAGGACTTGGG

339 GACCCCAGACCGCCTCCCTTTGCCGCCGGGGACGCTTGCTCCCTCCCTGCCCCCTACACGGCGTCCCTCAGGCGCCCCCATTCCGGACCAGCCCTCGGGA

+470 +525

439 GTCGCCGACCCGGCCTCCCGCAAAGACTTTTCCCCAGACCTCGGGCGCACCCCCTGCACGCCGCCTTCATCCCCGGCCTGTCTCCTGAGCCCCCGCGCAT

539 CCTAGACCCTTTCTCCTCCAGGAGACGGATCTCTCTCCGACCTGCCACAGATCCCCTATTCAAGACCACCCACCTTCTGGTACCAGATCGCGCCCATCTA

FIG. IC

639 GGTTATTTCCGTGGGATACTGAGACACCCCCGGTCCAAGCCTCCCCTCCACCACTGCGCCCTTCTCCCTGAGGAGCCTCAGCTTTCCCTCGAGGCCCTCC

739 TACCTTTTGCCGGGAGACCCCCAGCCCCTGCAGGGGCGGGGCCTCCCCACCACACCAGCCCTGTTCGCGCTCTCGGCAGTGCCGGGGGCGCCGCCTCCC

    Met
839 CCATG

FIG. 2A

-2277 AAGCTTTTACCAATACCTCCCGTCTTACCCCTCCTGGGCTTTGGGGAAATTAAAGTAGCCTCTTATGAGTAAGTCAG

-2200 GGGTCTCAGGTCTAAGGAGTTGTTAAGTGAGCAATAGGTACTCAGTAAACTAAGTATTATGAACAAAAGTGTGTATGTCTATGTCAGGAAGAGGGGTGGC

-2100 CATCAGAATTTATGGCTTGCGCTGTTTCCTAGAAGTGATGTAATGAACTTTTGCTACTCTATCCACACTCTAATCTGAATCTACTTAAGGTGCATCAGTG

-2000 TCTGTACCAAGAAGGTTGTCTATAAACATGAAAGATGGATGCTCACTGGCTTGTGGAAGCTGAACCTGTATCCTCAGAAAATACAGTGATAGCTAATTCA

-1900 GGTAACCAGCCATATTCCACAGCAGCATCTTCTCTCAGTAGCTCTGGTTTGGAGCTCCTGCTCTGTGTCTATAATGGCCACAGGTGTAAGAATATTCACT

-1800 TTTTGTCCAATCTGTAGAGCTAGCCTACTGCAGTTCTCAAACTGAACTCAGAGGGAGGACCTAACTGGATGAAACTACTAGTCTGACAGTAGCGCCTCTT

-1700 GATTATCTTTTTCTTGGGCTACTGGGATGGTAGCTTTGCTTCAACTCAAAACTGGTATCAAGGAAAGGAACCTGCTGGTGCTGATTTATACATAATTTTT

-1600 AGAATTATTCAGAAGTGGGTTGGAACAATTATTTTATTCCAGAGTTTTTCAATGTGTGATAATGGAAAAAATTCTGTATTCAAGGGAGTTTGGAAAATGC

-1500 TGGGTTAAAAGAGTGAAAAGGTTTTCTCTTCTACAGGAGTTTCAGAGCCTTTAACATGATAATGTTCCAGAATGAGGAATCTAAGAGGACAGGAGAGTAC

-1400 CCAGTATCTCCCAAACTTGTTTGACTCCAGAATTCCTGTTTGTCAGAACATATTCTGGGACCATTGTTTCTCAGAAGTACATAGTAGGTAAGAACATAGT

FIG. 2B

```
-1300 GGATCCTGACTGCAAAAATCCAGCTCTACCACTTACTGTGGTCTCGAACAAAGTACTTAACTTCTTTGTACCTCAGTCTCCTCATCTGCCAGATATGGAT

-1200 AATAAGACCCACTTTATAGGTTCATAGTGAAGATTAAATGACCATACACAACACACATCAAATTACTAAGTGTAGTATATGTTAGCTATTATTATTTTAT

-1100 TTATTCAGTGCTCTACTAATAACCTAGGCCCCATACACAACTGAAGTATAATTCCAAAAGTGATAGAAAGTTCTTTGTGACTTTTCTGAACTCAGGAACA

-1000 TCTGAAGTAGAGAACAGTATAGAGATCTTGGGTTTGGGAGTACATTCAACAGAGTTTTCCAGTTTAAATCATCTGTCTGGTCAGTATGGCTGCAGAGTCA

 -900 TGCCGAAATGAAAATGTTGACTTTGAGTAACTAAAGGTAAAATAAAAGAAAAAGGGAAGGTGGAACAGTGGTAAGAGTTATTCTGTATTCATCTAATTTA

 -800 AGACTTAGTTGAAATTGAAAATGTCAAGTTATGAGTAGTGTAGAACAAGTAGACATCAAACACTTAAAATTCCAGCTTCCTGGATTATGCTATGGAAAGA

 -700 ATGAAGTTGGTGGATAATGTTTAGCCTAGCAAGAAGGTCAAGAAGAAGAAAGCCATACAAGAAGTGGCTTAGGCAGCAAATTATAAAGGTGACCATTCAT

 -600 TCAAATCAGTAAAACAAACAAGTATACCTTATTCTTTAGGTAAAATTGATGGATCTCTGTTTTCCAGCAGTTCACAAACAGAGGGGTACATTGTAAACAA

 -500 CAAACTAACAAAATAAATTCTGGGATGGCAACCTGCTAAGGTATCCCAGAAAATAAGAGGTAGGACATGAATTTAAAAGATTGGAAGGTATGTCTTCAGT

 -400 ACTGGCCTGGCCCTGAGTAGACTAGTGCCCTCCATAGGGGTGCGTGTGCACACATAATACAGGAGGGAAGCCTTCCCTTCTAGAGCAAGTGATTCAGCTT
```

FIG. 2C

-300 GGGAGGCTGTGACTGAGCTACACTAAGTAAAAACGGGAGACTTGATTGTCCTTCAACAGACCTGTCCAAAATGACTGGAAAGTAAATACCGTAAATCACT

-200 GTTGTCAGGGCGCACATTCCACCTCCTTCCTCCCTTACCCACAGCGGTCCACATTTCCACACTCCCTACACGGTTCGGGGAGAGCTCGTGGTCTAAGTAA

-100 CGAGAGGACTTCTGACTGTAATCCTAGCACGTCACTTTGTTGAAGGCAGACACGTGGTTCAGAGAGAACT$\boxed{\text{TATAAA}}$TCTCCCCTCCCCGCGAAGATCGTG

-1 ATGTTATTCGCTGGCAGCAGAAGGTCTTGCCGAGCGAGCTCCAGAACGTCCTGACAAGAGAAAGACAGATTGAGATAGAGATAGAAAGAGAAAGAGAGAA

101 AGAGACAGCAGAGCGAGAGCGCAAGTGAAAGAGGCAGGGGAGGAGGGGATGGAGCATATTACGTGACCGGCCTAGGGAGTCATCCAGGAACAAACTGAG

201 GGGCTGCCCGGCTGCAGACAGGAGGAGACAGAGAGGATCTATTTTAGGGTGGCAAGTGCCTCCTACCCTAAGCGAGCAATTCCACGTTGGGGAGAAGCCA

301 GCAGAGGTTGGGAAAGGGTGGGAGTCCAAGGGACGCCCCTGCGCAACTCCCTCAGGAATAAAACTCCCCAGCCAGGGTGTCGCAAGGCTGCCGTTGTGA

401 TCCGCAGGGGGTGAACGCAACCGCGACGGCTGATCGTATGTGGCTGGGTTGGCGTTTGGAGCAAGAGAAGGAGGAGCAGGAGAAGGAGGGAGCTGGAGGC

FIG. 2D

501 TGGAAGCGTTTGCAAGCCGCGGCGGCAGCAACGTGGAGTAACCAAGCGGGTCAGCGCGCGCGCGCCAGGGTGTAGGCCACGGCGCGCAGCTCCCAGAGCA

601 GGATCCGCCCGCCCTCCGCAGCCTCTGCGGCCCCTGCGGCACCCGACCGAGTACCGAGCGCCCTGCGAACGGCACCCTCCTCCCCGCGGTGGCTGGGCTC

701 GCCCCAGCGCGCACACGCACACACACACACACACACACACGCACGCACACACGTGTCGTTCTCTGCTCCGGAGCTGCTGCTGCTCCTGCTCTCAGCGC

801 CGCAGTGGAAGGCAGGACCGAACCGCTCCTTCTTTAAATATATAAATTTCAGCCCAGGTCAGCCTCGGCGGCCCCCCTCACCGCGCTCCCGCCCCTCCCG

901 TCAGTTCGCCAGCTGCCAGCCCCGGGACCTTTTCATCTCTTCCCTTTTGGCCGGAGGAGCCGAGTTCAGATCCGCCACTCCGCACCCGAGACTGACACAC

1001 TGAACTCCACTTCCTCCTCTTAAATTTATTTCTACTTAATAGCCACTCGTCTCTTTTTTTCCCCATCTCATTGCTCCAAGAATTTTTTTCTTCTTACTCG

1101 CCAAAGTCAGGGTTCCCTCTGCCCGTCCCCTATTAATATTTCCACTTTTGGAACTACTGGCCTTTTCTTTTTAAAGGAATTCAAGCAGGATACGTTTTTC

1201 TGTTGGGCATTGACTAGATTGTTTGCAAAAGTTTCGCATCAAAAACAACAACAACAAAAAACCAAACAACTCTCCTTGATCTATACTTTGAGAATTGTTG

Met His Tyr Cys Val Leu Ser Ala Phe Leu Ile
1301 ATTTCTTTTTTTTATTCTGACTTTTAAAAACAACTTTTTTTTCCACTTTTTTAAAAAATG CAC TAC TGT GTG CTG AGC GCT TTT CTG ATC

FIG. 3A

```
  1 CAGTAGTAGC TTCCAGAACT TGCTTAGCAC CTGAATCACG TGTGAGGTTT
 51 GTAAAGAAAC AGAGATGCCA GGGCCTCAGC TCTGGAGACT GATTGGTAGA
101 GGTGGAGTCC AAAAAAGTAT AACTTTAATA ATTTTCCTTC CTATCTTCAA
151 CTGTCTGCTC AAAGGCCTTC CCTTATCACC CTATTTGAAA CTGCAACATC
201 CCCCAACCTA GGCACACCCC ATCCTCCTTC CCTGCTTGAT TTTCTGCCAC
251 ACCACATTTG TTTGTTTGCT TGTCTGTTTG AGACACGGTC TTGCTCTGTC
301 GTCCAGGCTG GAGTGCAGTG GTGCAATCTT GGCCCCCTGT AAACTCGCCT
351 CCCTGGCTCA AGTGATTATC CTGCTCAGCC TCCCAAGTAG ATGCGTGCGC
401 CAACATGCCG GGCTAATTTT TCCATTTTTT TGTAGAGACT GGGTTTCGCC
451 GTGTTGCTGG GGCTGGTCTC GAATTCCTGA GCTCAAGTAA TCCTCCTGCA
501 TGGGCCTCCC CAAATGCTGG GATTACAGGC GTGAGCCACT GCACCTGGCT
551 CAGCACTTTT TACCGTACTA CATCATTTAC ATATTTATTT AGTTTATCGC
601 CTCCTCCACT GCCCCACCCC TGCCTCTAAA TAAAATTTCC CTGAGGGCAG
651 GAGTTTTGTT TCGTTCACTG ATATTCTTCA CAGAGCCTAG AATAGTGCCT
701 GGTATATAGA AACATTAAAC TTTTTCTGAA ATTTCAGAGG CAGTATAGCA
751 TAGTAATTAA GTCCAGAATC TGGCAACGTC CTGGGTGCAA ATCCCAACAG
801 CTGACACCTA ATAACTATGT GACCTTGGGC AAGTTACTTT TAAAGTTTCT
```

FIG. 3B

851 ACCCCTAGGT TTCCCATTGG TTTTGCAAAT GAAAGTAATG CCTACCCAAG

901 CTAGATAGCC TCTGTAAATA TCGCCTCCAT CACTCACAAG CAGTGTGGTC

951 TGTAAAAAAA AAAACAAAAA ACTCTATGCC TCAGTTTCCT CATCCGTAAA

1001 AGTGACCCAC CGCTGTGCTG GGATACAGAG AACAGCCCCT TCAGTTAGTG

1051 GCCTGGAAGC CAGCCTCTCA GAAAGGGTCC AGGAAGGCTG GAGTGAGATG

1101 GGGTGGAGCG GCACTCACTC TCAGGAAAGT TCAGTTCAGA GGCAAGCCCT

1151 GTGTTGCGGG GTGCGGGGAG CCACGTGCCC TACCCTCCCT TGGCTGCTCG

1201 TGGGAAAAGG CCTAGAGGTT CGGGCCGAGA AGAGGAGCGA AAGCACAGAG

1251 CCGACTTCCC CTCACCCATC TGGGAAATGG CTCGGGCCAA CTGCTGACTT

1301 CGCGCTCGCT GGCCGACGTC CTGCGGAGAC CTCGGCGGGG AGGGAGGCTG

1351 AACATCTGGA TGACATTTCT GCGAGAGAGC GGCTCCGGAG CGGCGGTCGG

1401 GGAGGGAGAG CTGCTCGTGC GCACGTCGGG CCGGGAGGGA GGCGATTCCT

1451 CGGGGCCTGG GTCTTGTTTT TCTCGCTCTC TACCGCAGCC CCTTCTCCCG

1501 CCCCTCAGCC CCCACCCCGC AGCCCCCAGC CCCCGAGCCT CCCCGGCTCC

1551 CGACCAGCCG AGCTCCTTCA CTGGCGGCCT CCGCTCGCCA GAGGGCACCC

1601 TCGATCTTCC GGAAAACGCC ACCATTTTTC ACTGCCCCTG GAGCGTCTCC

1651 AGGCTTCTGC CCGCCTCCCG ACTCCGATCT TGTCAATGAA GAATCGGGCC

FIG. 3C

```
1701 AGGATCGCCG CGGAGCGGAC GCCGACCCTC CGACCCGGCT CGCAGGCTGG

1751 GAGTCCCCTC TGCGAGGCTG GCATGGCCGC CCCTACCGGG TCCCGCGCCC

1801 TCTGCGGACC CTCCCCGGGT TGGGCCTGGC CGCGGGCGGC CCCGGGACCG
                                                   -301
1851 GGGGACCAGG AGGGAGAGTA GACCGGGCCG GACGGCGCGG ACTGACAGCT

1901 GGCGAGAGGG CGCCGGGGCT GGGGGAAAGG GAGGGAGGGG GCTCATCGGA
                                -221
1951 GTAACTTTCC AGAAAAACAC CAACGTGTGG CAGGAGTGAT TCCAAGAGGG

2001 GAAAAAAAGT TCAGCTACCA CGTCGAACGA GAGGACTCGC AAAGTATTTT

2051 TCAAAAGGGC TCGGCTTTTC CTGTGCCTGT TTAAAACATT AACATCGTGC
        -91                                  -60          -47
2101 AGCAAAAGAG GCTGCGTGCG CTGGTCCCTC CCTCCCCCAC CCCAGGCCAG
           -38                                           +1
2151 AGACGTCATG GGAGGGAGGT ATAAAATTTC AGCAGAGAGA AATAGAGAAA
                                          +35
2201 GCAGTGTGTG TGCATGTGTG TGTGTGTGAG AGAGAGAGGG AGAGGAGCGA
                                 +75
2251 GAGGGAGAGG GAGAGGGAGA GAGAGAAAGG GAGGGAAGCA GAGAGTCAAG
        +110
2301 TCCAAGGGAA TGACCGAGAG AGGCAGAGAC AGGGGAAGAG GCGTGCGAGA

2351 GAAGGAATAA CAGCAGCTTT CCGGAGCAGG CGTGCCGTGA ACTGGCTTCT

2401 ATTTTATTTT ATTTTTTTCT CCTTTTTATT TTTTAAAGAG AAGCAGGGGA

2451 CAGAAGCAAT GGCCGAGGCA GAAGACAAGC CGAGGTGCTG GTGACCCTGG

2501 GCGTCTGAGT GGATGATTGG GGCTGCTGCG CTCAGAGGCC TGCCTCCCTG
```

FIG. 3D

2551 CCTTCCAATG CATATAACCC CACACCCCAG CCAATGAAGA CGAGAGGCAG

2601 CTGAAAAAGT CATTTAGAAA GCCCCCGAGG AAGTGTAAAC AAAAGAGAAA

2651 GCATGAATGG AGTGCCTGAG AGACAAGTGT GTCCTGTACT GCCCCACCTT

2701 TAGCTGGGCC AGCAACTGCC CGGCCCGCTT CTCCCCACCT ACTCACTGGT

2771 GATCTTTTTT TTTTTACTTT TTTTTCCCTT TTCTTTTCCA TTCTCTTTTC

2801 TTATTTTCTT TCAAGGCAAG GCAAGGATTT TGATTTTGGG ACCCAGCCAT

2851 GGTCCTTCTG CTTCTTCTTT AAAATACCCA CTTTCTCCCC ATCGCCAAGC

2901 GGCGTTTGGC AATATCAGAT ATCCACTCTA TTTATTTTTA CCTAAGGAAA

2951 AACTCCAGCT CCCTTCCCAC TCCCAGCTGC CTTGCCACCC CTCCCAGCCC

3001 TCTGCTTGCC CTCCACCTGG CCTGCTGGGA GTCAGAGCCC AGCAAAACCT

3051 GTTTAGACAC ATGGACAAGA ATCCCAGCGC TACAAGGCAC ACAGTCCGCT

3101 TCTTCGTCCT CAGGGTTGCC AGCGCTTCCT GGAAGTCCTG AAGCTCTCGC

3151 AGTGCAGTGA GTTCATGCAC CTTCTTGCCA AGCCTCAGTC TTTGGGATCT

3201 GGGGAGGCCG CCTGGTTTTC CTCCCTCCTT CTGCACGTCT GCTGGGGTCT

3251 CTTCCTCTCC AGGCCTTGCC GTCCCCCTGG CCTCTCTTCC CAGCTCACAC

3301 ATG

FIG. 4

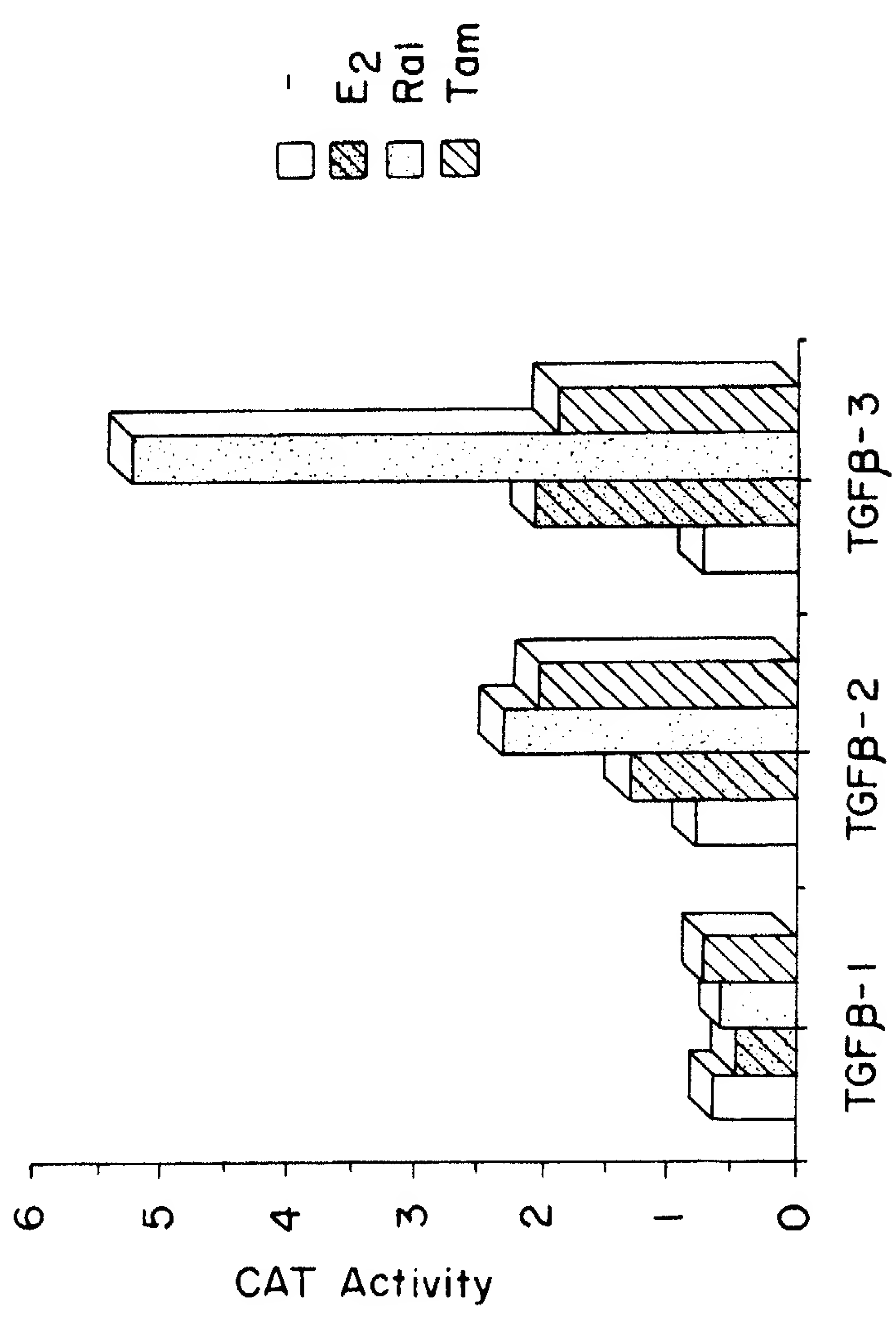


FIG. 5A

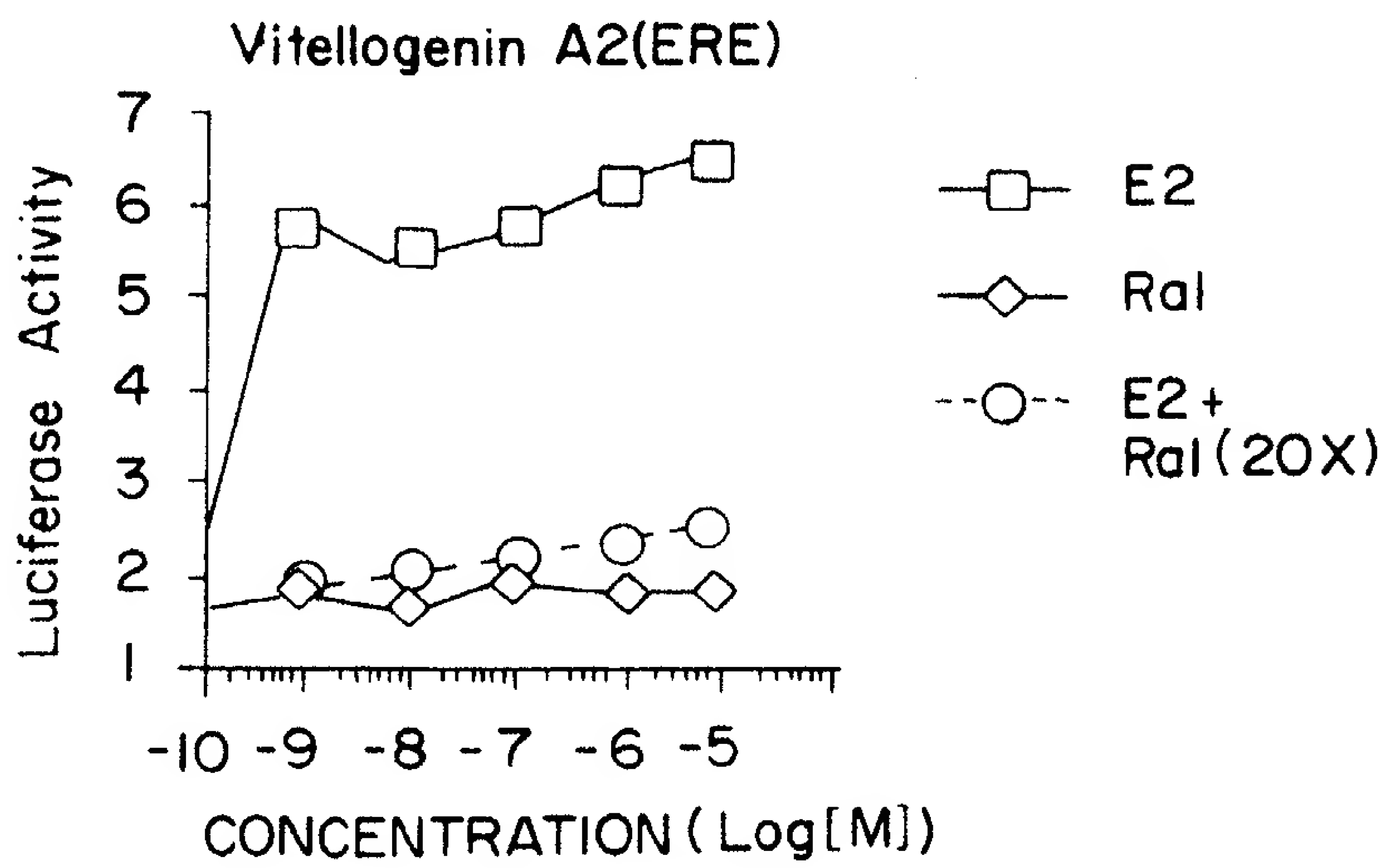

Vitellogenin A2(ERE)
Luciferase Activity
7
6
5
4
3
2
1
-10 -9 -8 -7 -6 -5
CONCENTRATION (Log[M])
E2
Ral
E2 +
Ral (20X)

FIG. 5B

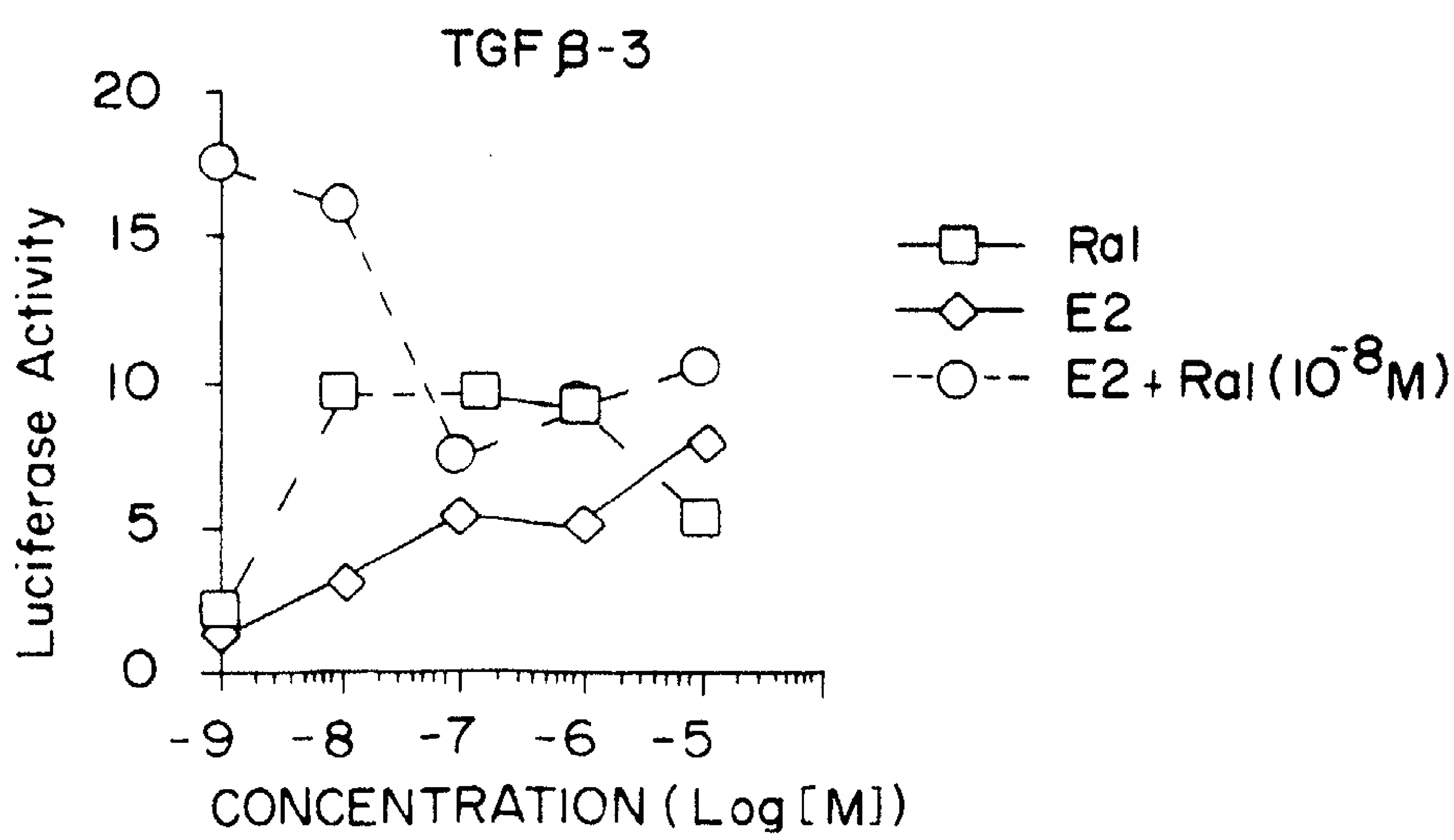

TGF β-3
Luciferase Activity
20
15
10
5
0
-9 -8 -7 -6 -5
CONCENTRATION (Log [M])
Ral
E2
E2 + Ral (10⁻⁸M)

FIG.6

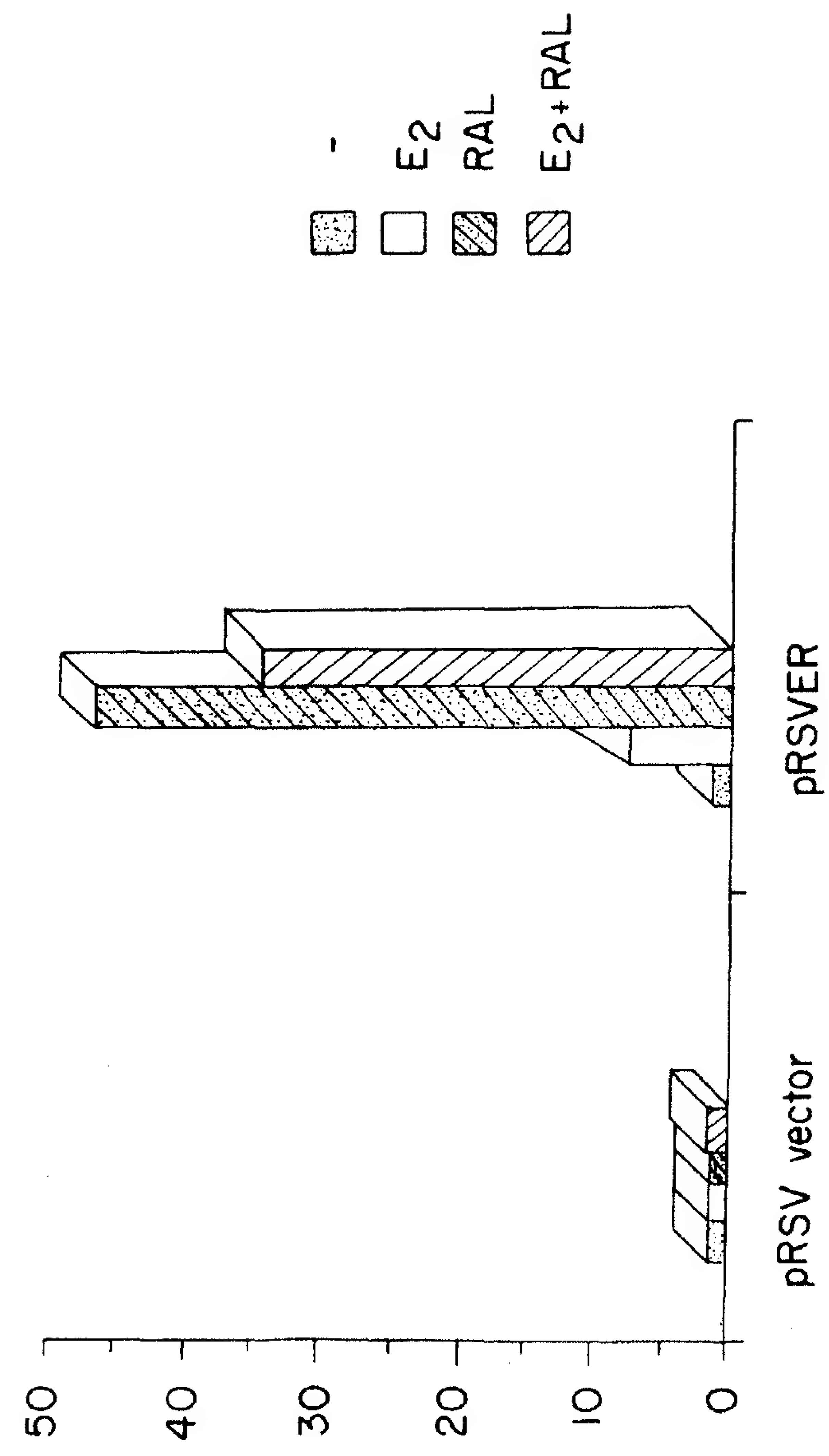


FIG. 7

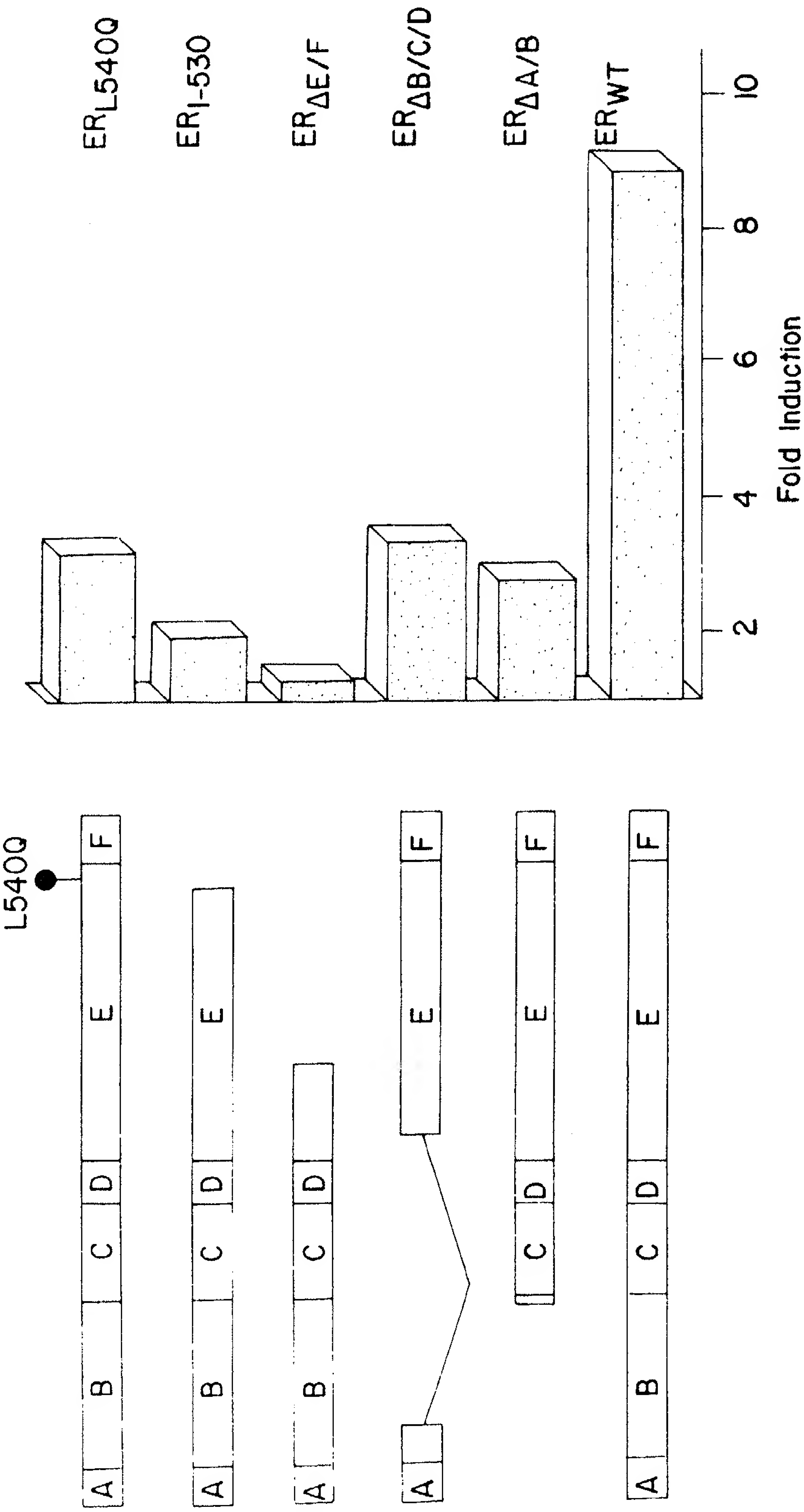

ER L540Q
ER 1-530
ER ΔE/F
ER ΔB/C/D
ER ΔA/B
ER WT
2
4
6
8
10
Fold Induction
L540Q
A
B
C
D
E
F

FIG.8

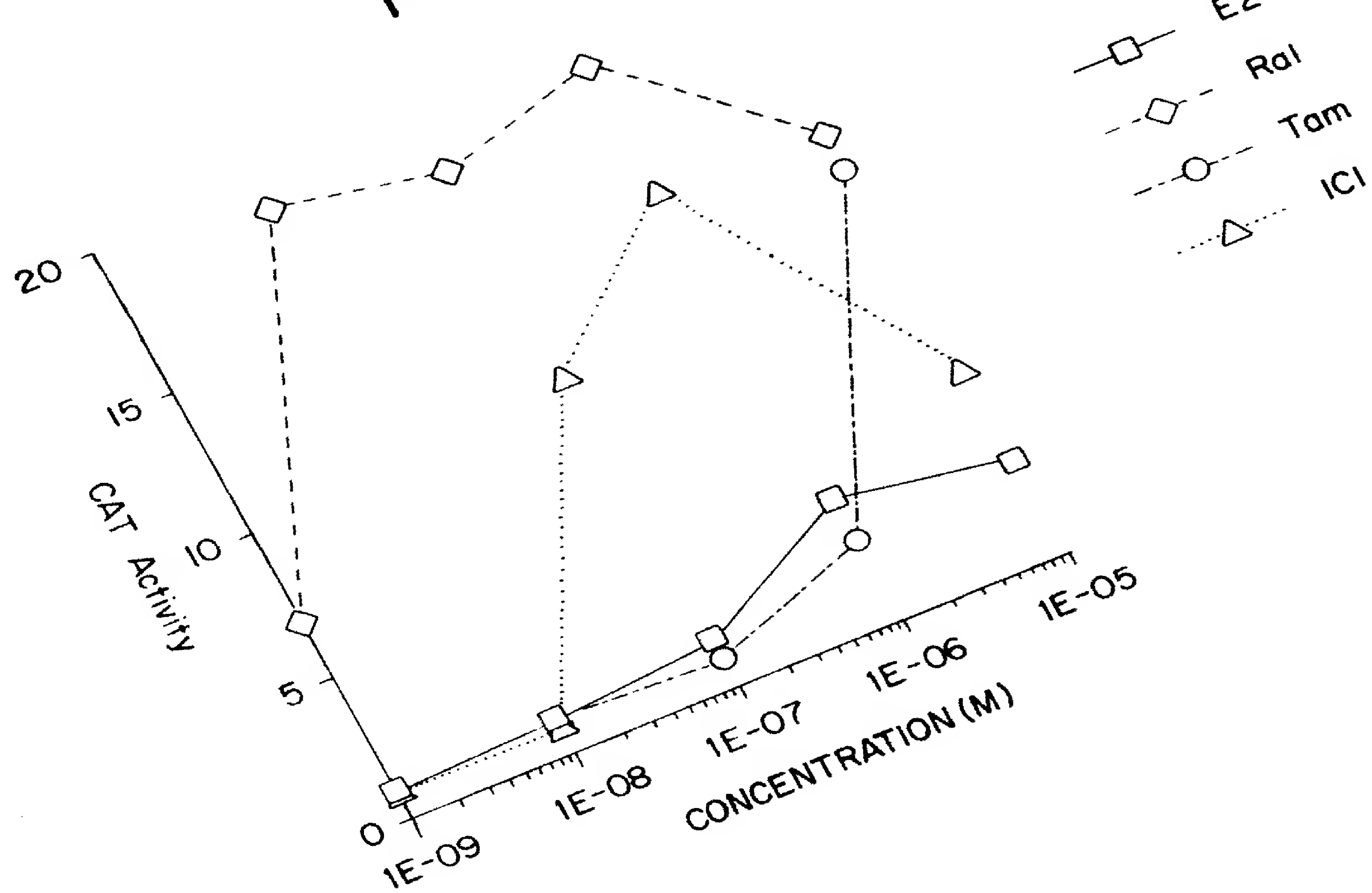

20
15
10
5
0
CAT Activity
1E-09
1E-08
1E-07
1E-06
1E-05
CONCENTRATION (M)
E2
Ral
Tam
ICI

FIG. 9

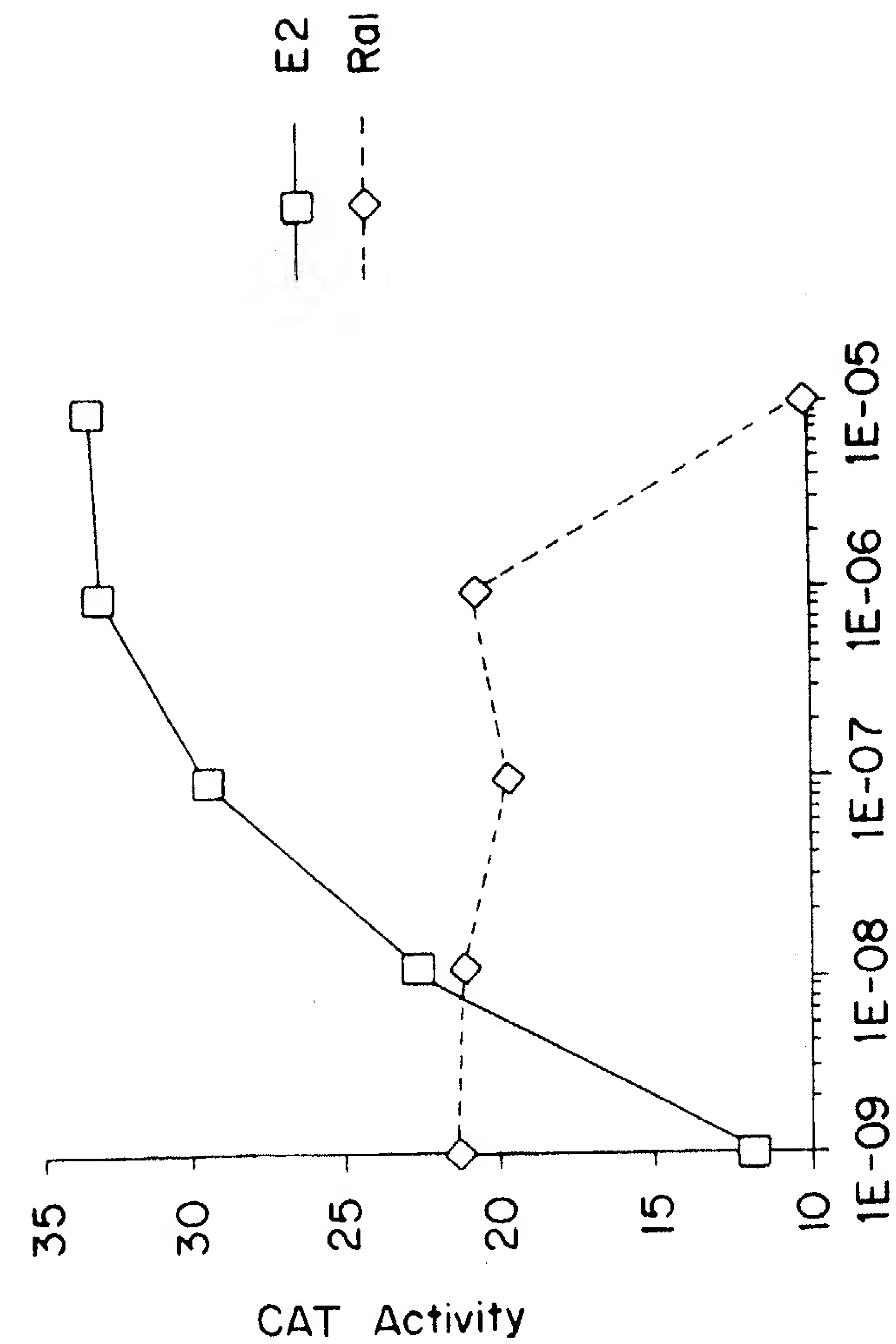

E2
Ral
CAT Activity
35
30
25
20
15
10
1E-09
1E-08
1E-07
1E-06
1E-05
CONCENTRATION (M)

FIG. 10

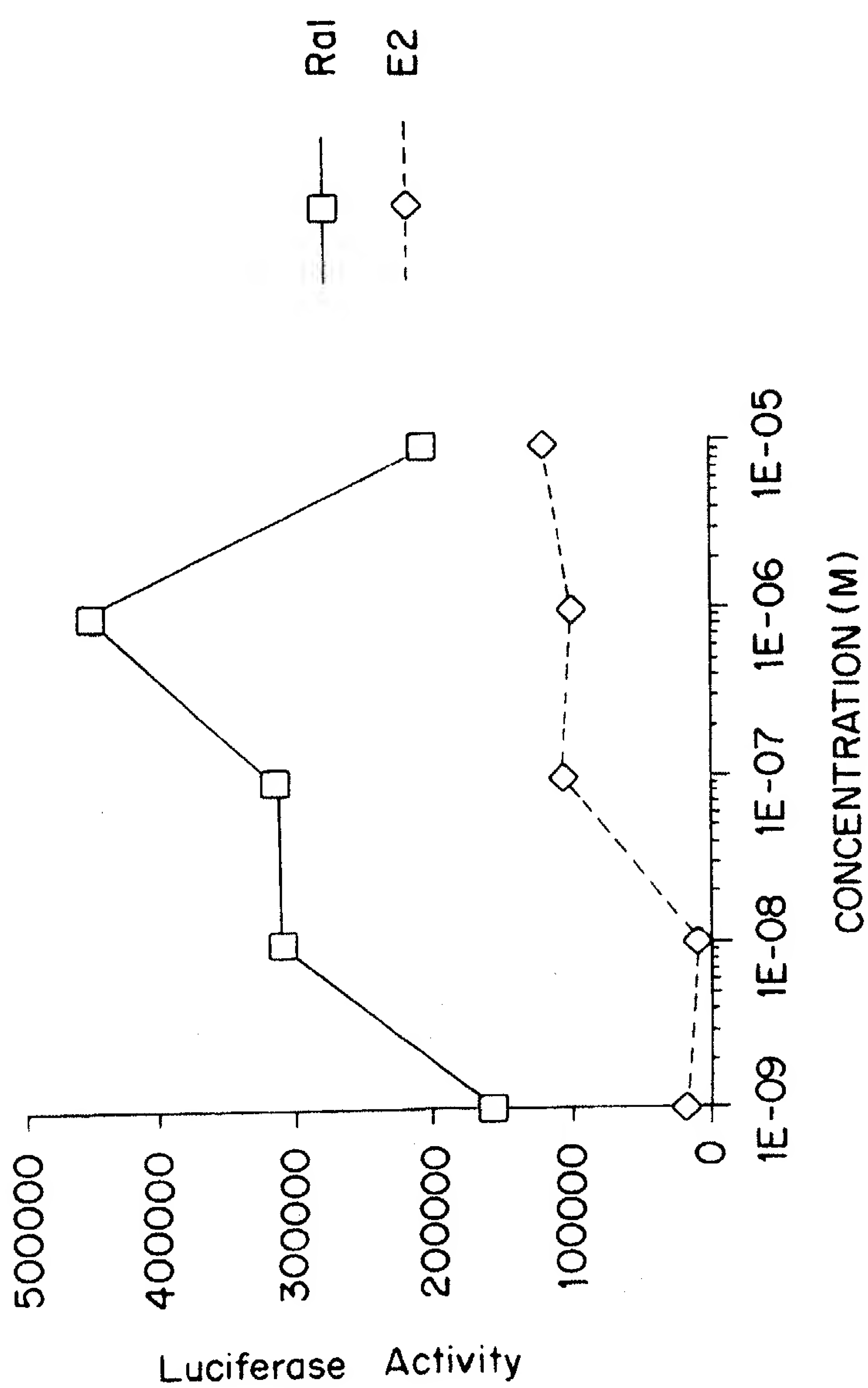
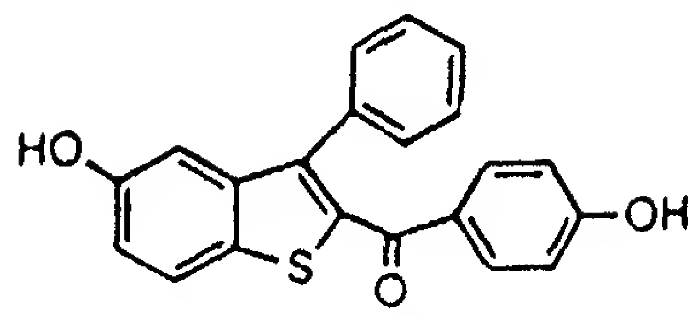
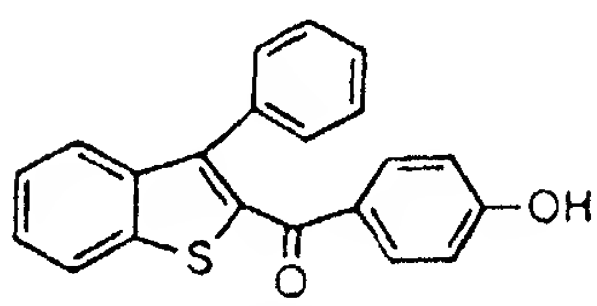

Ral
E2
Luciferase Activity
500000
400000
300000
200000
100000
0
1E-09
1E-08
1E-07
1E-06
1E-05
CONCENTRATION (M)

FIG. II

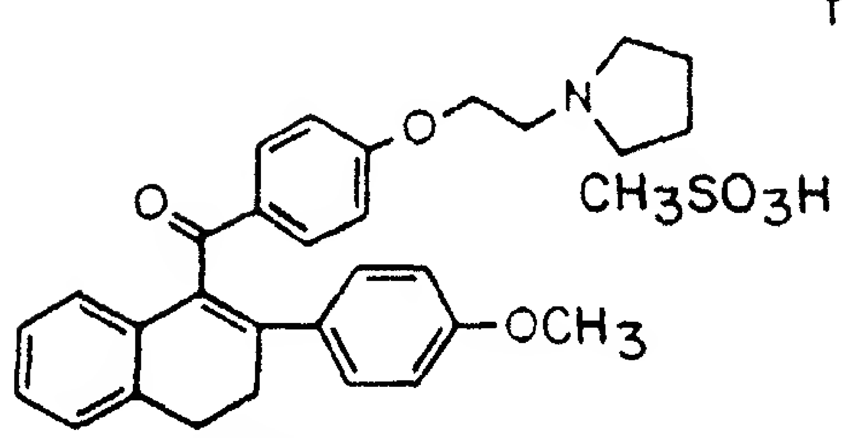
Estrogenic



112676

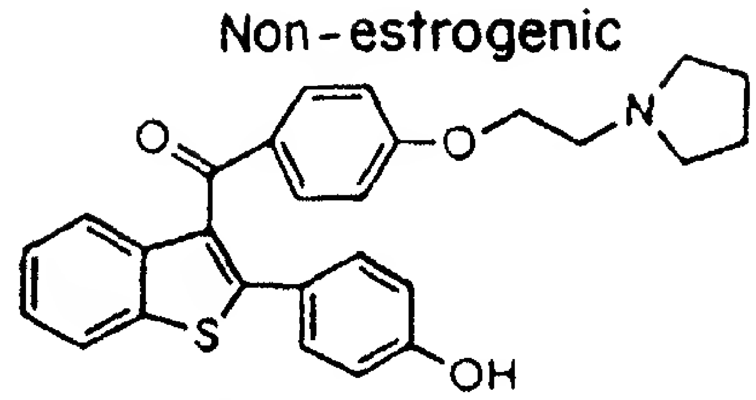


81099

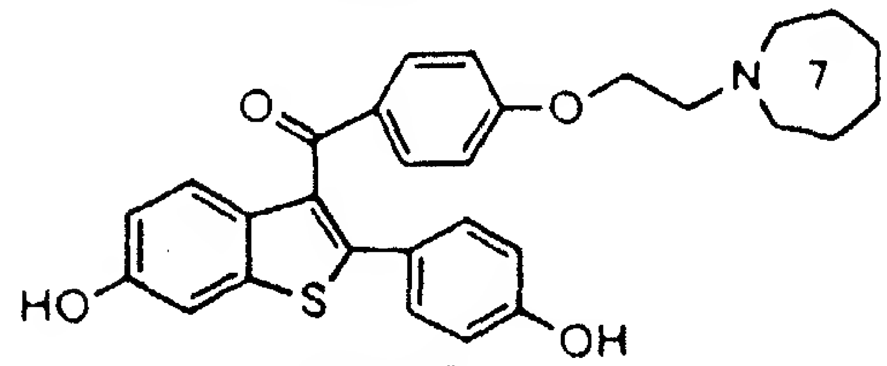


133314

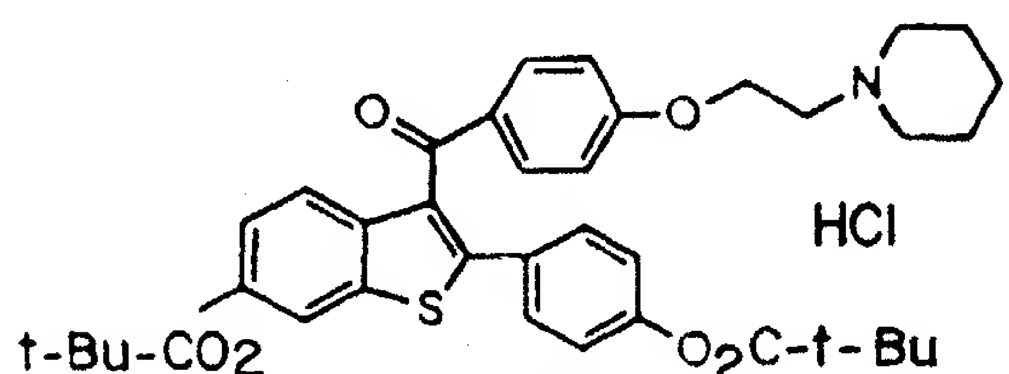
Non-estrogenic



113526

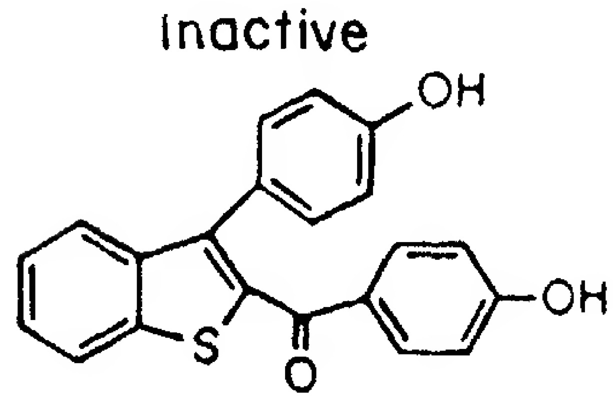


139482



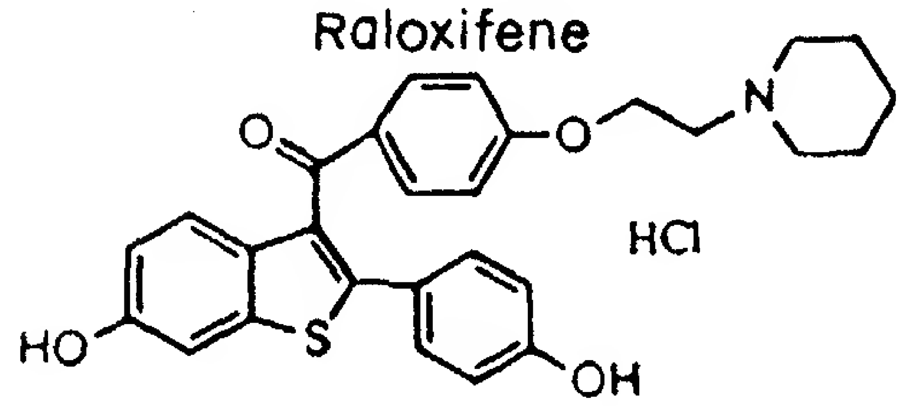
177366

Inactive



98005

Raloxifene



156758

FIG. 12

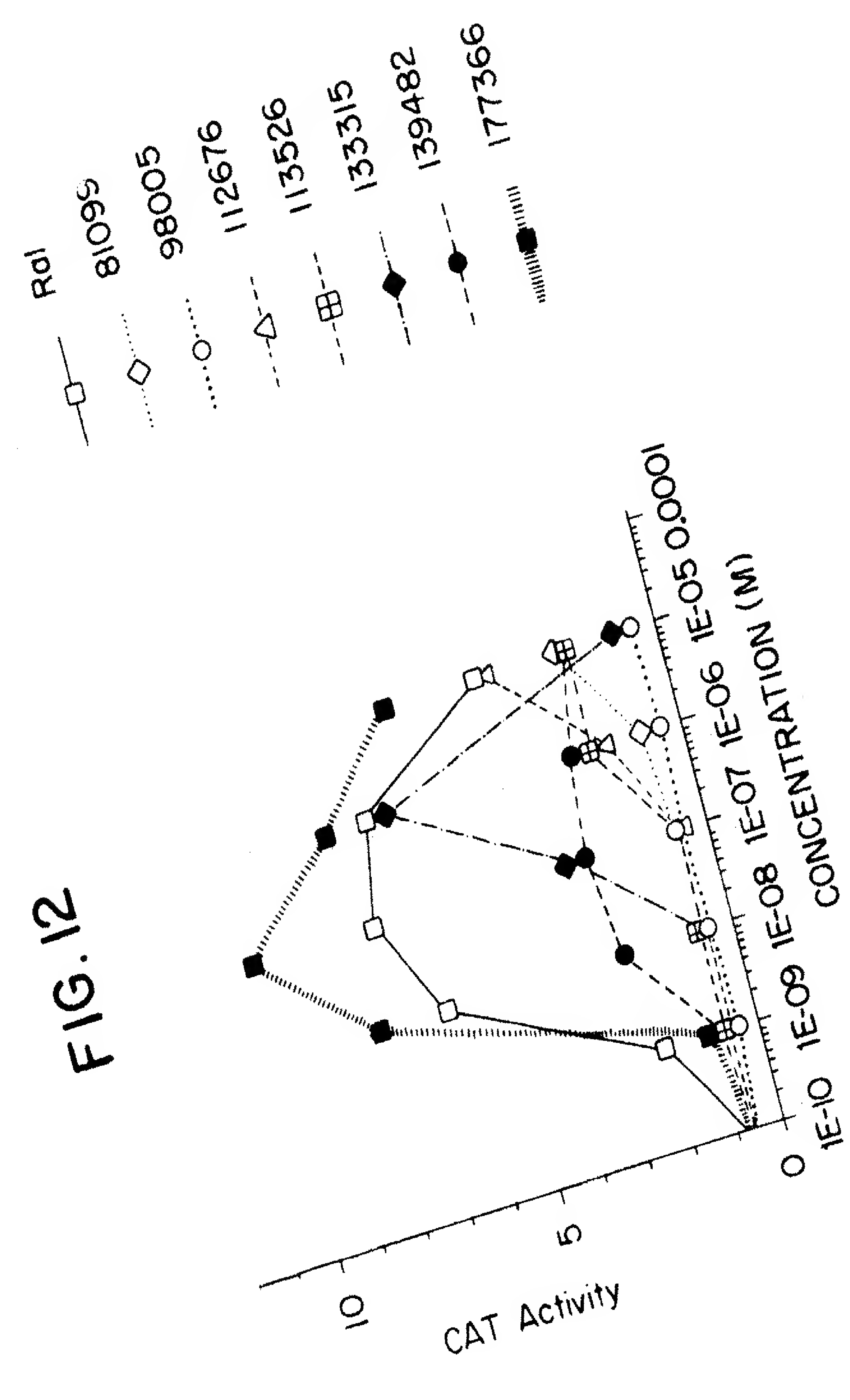

Ral
81095
98005
112676
113526
133315
139482
177366
CAT Activity
0
5
10
CONCENTRATION (M)
1E-10 1E-09 1E-08 1E-07 1E-06 1E-05 0.0001

FIG. 13

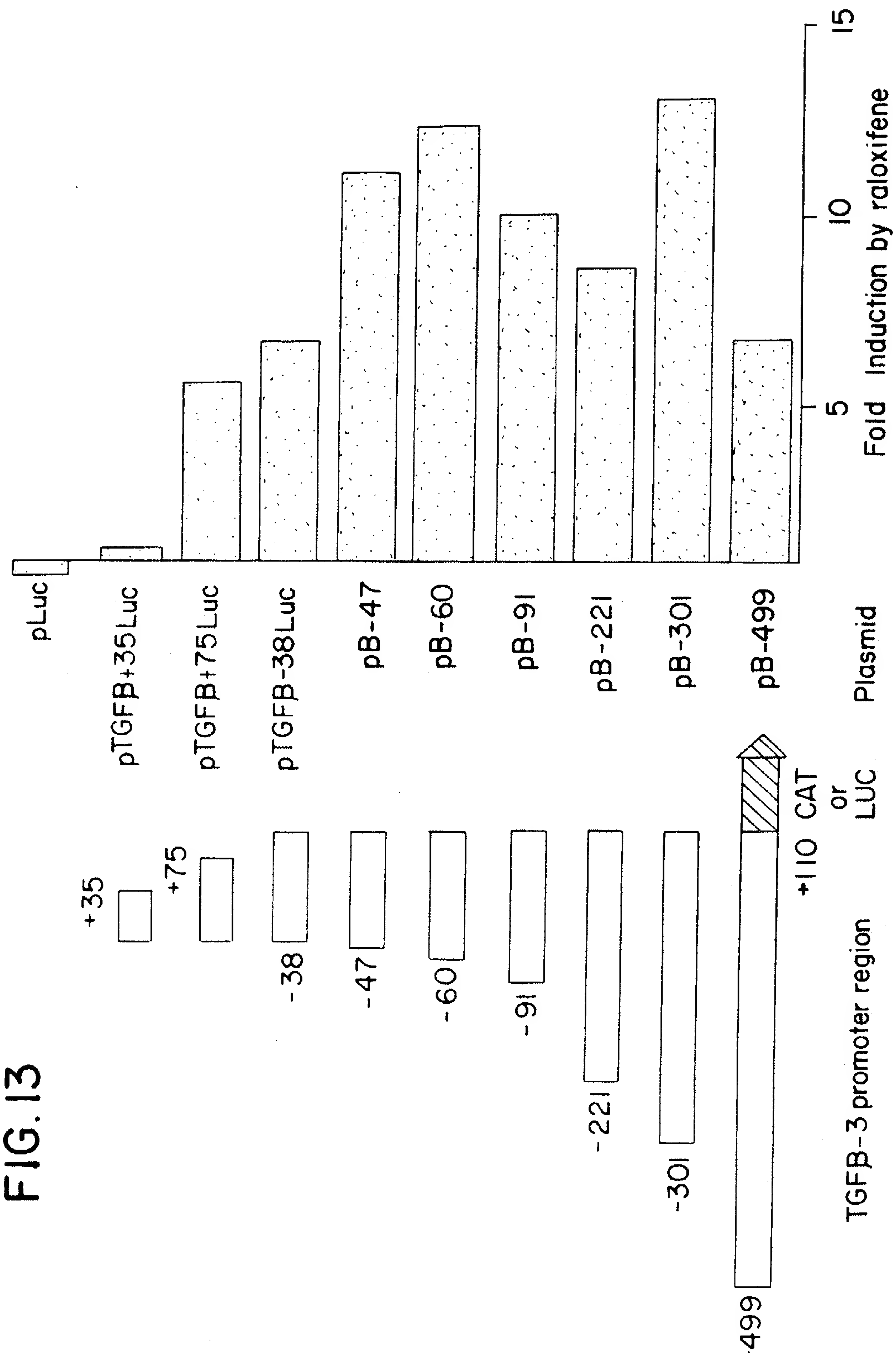

Fold Induction by raloxifene
5
10
15
Plasmid
pLuc
pTGFβ+35Luc
pTGFβ+75Luc
pTGFβ-38Luc
pB-47
pB-60
pB-91
pB-221
pB-301
pB-499
+35
+75
-38
-47
-60
-91
-221
-301
-499
+110
CAT
or
LUC
TGFβ-3 promoter region

FIG.14

```
-38                                                          +1
TGGGAGGGAG   G[TATAAAA]TT   TCAGCAGAGA   GAAATAGAGA
                                          +35
AAGCAGTGTG   TGTGCATGTG     TGTGTGTGTG   AGAGAGAGAG
                                          +75
GGAGAGGAGC   GAGAGGGAGA     GGGAGAGGGA   GAGAGAGAAA
             CCCTC-motif
                            +110
GGGAGGGAAG   CAGAGAGTCA     AGTCCAAG
                ERE half sites
```

FIG.15

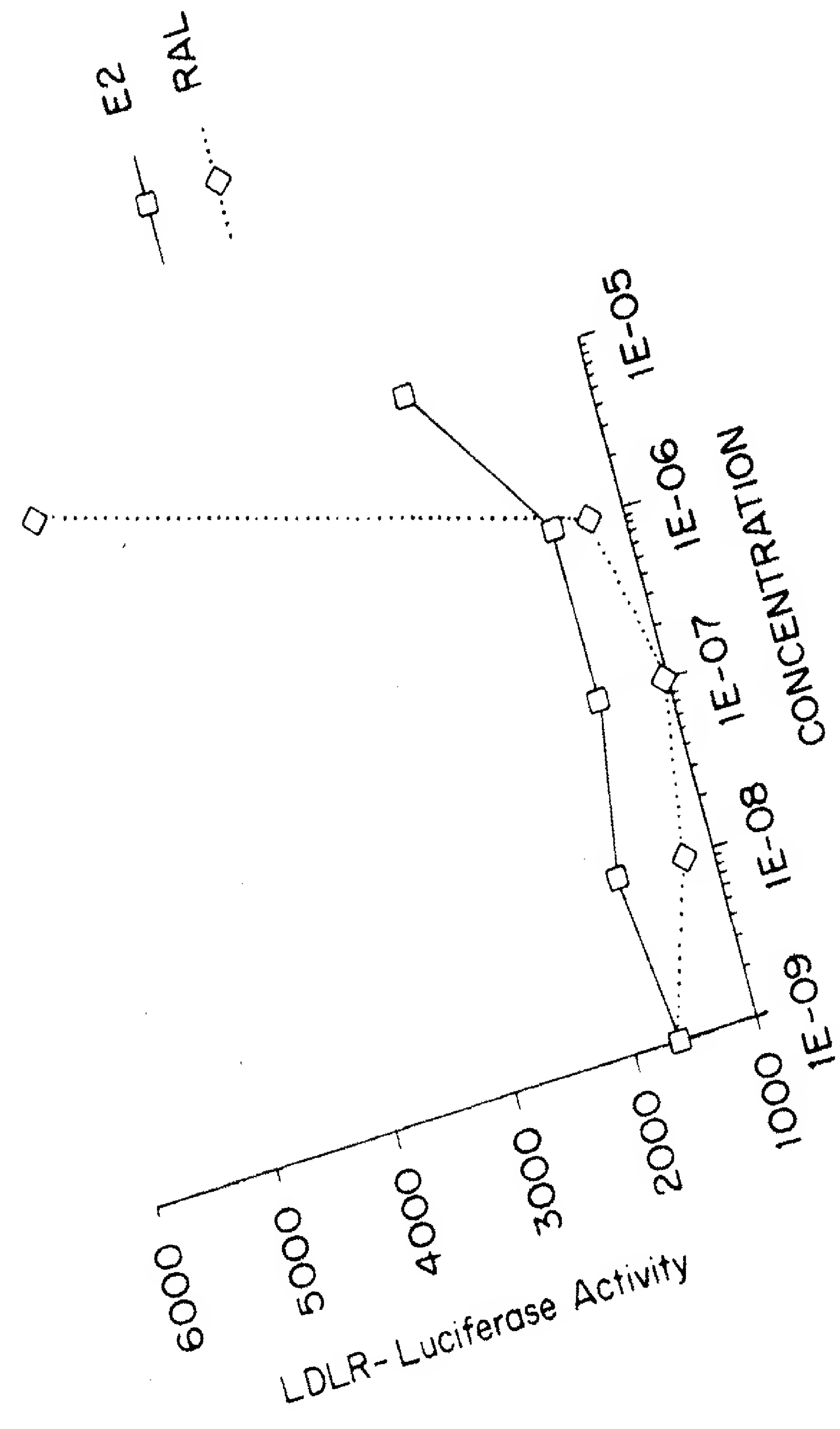

E2
RAL
LDLR- Luciferase Activity
6000
5000
4000
3000
2000
1000
1E-09
1E-08
1E-07
1E-06
1E-05
CONCENTRATION

FIG.16

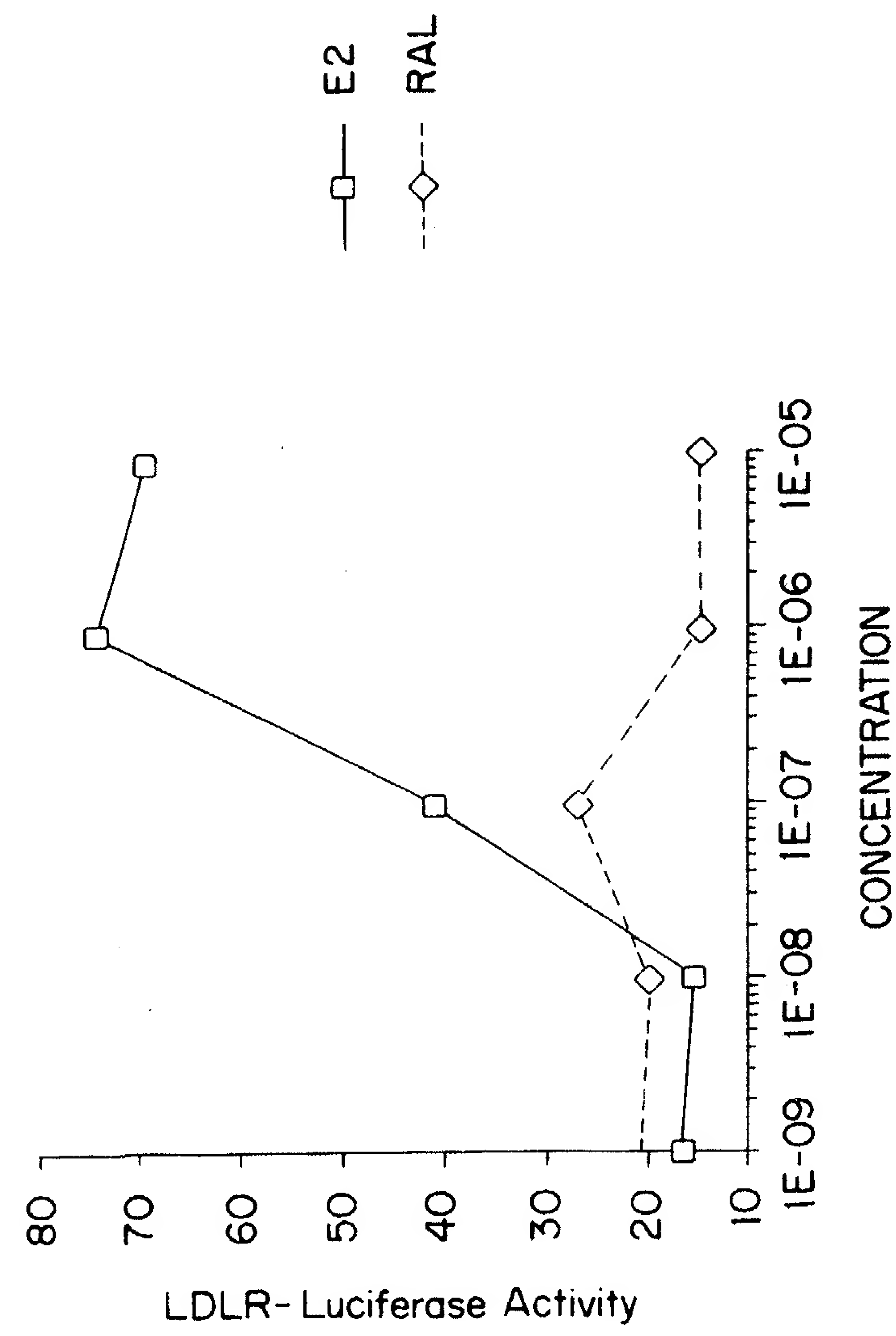
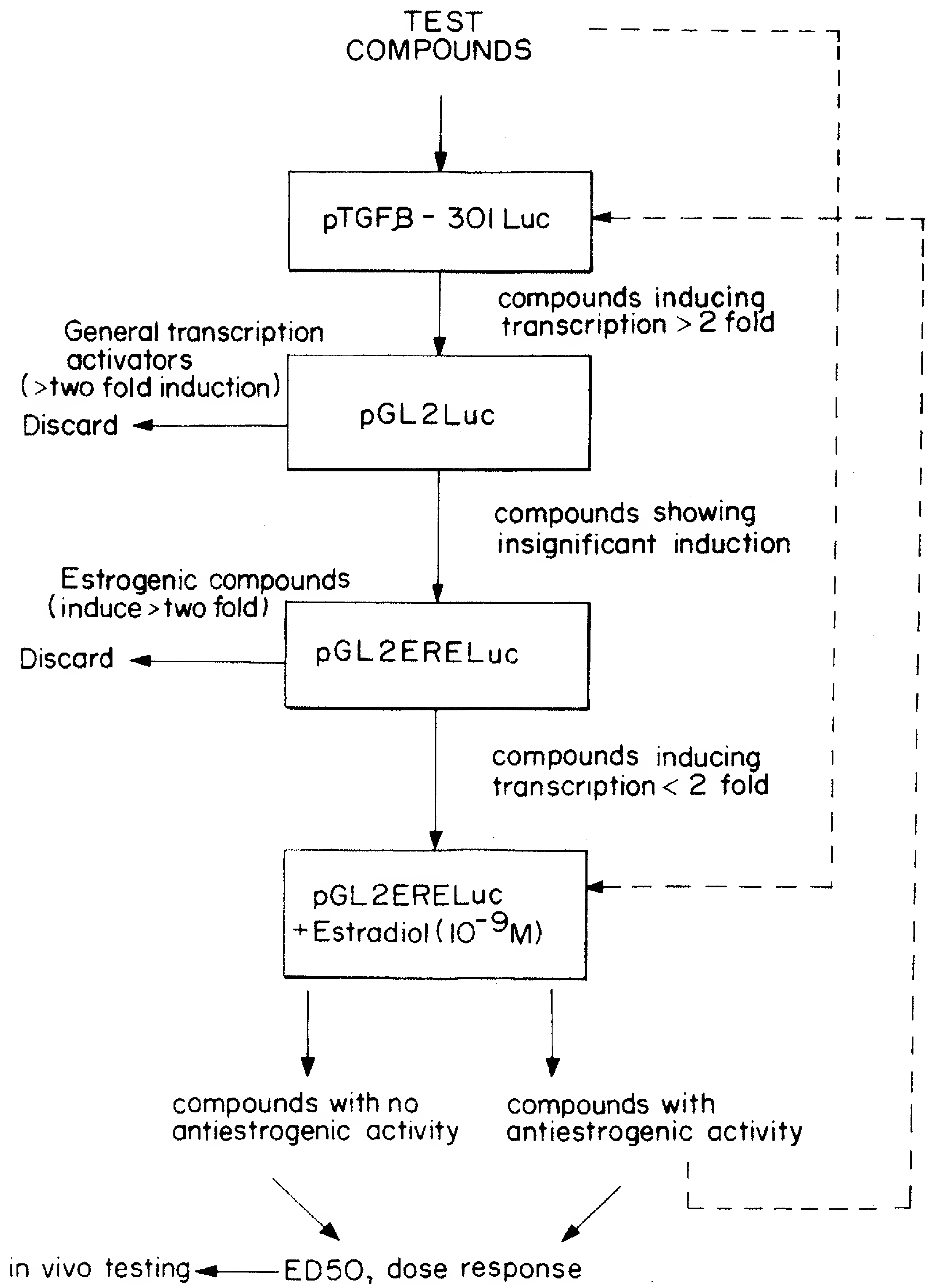

E2
RAL
80
70
60
50
40
30
20
10
LDLR- Luciferase Activity
1E-09
1E-08
1E-07
1E-06
1E-05
CONCENTRATION

FIG. 17


TEST
COMPOUNDS
pTGFβ - 301 Luc
compounds inducing
transcription > 2 fold
General transcription
activators
(>two fold induction)
Discard
pGL2Luc
compounds showing
insignificant induction
Estrogenic compounds
(induce >two fold)
Discard
pGL2ERELuc
compounds inducing
transcription < 2 fold
pGL2ERELuc
+Estradiol (10-9M)
compounds with no
antiestrogenic activity
compounds with
antiestrogenic activity
in vivo testing
ED50, dose response

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(54) Materials and methods for screening anti-osteoporosis agents.

(57) The present invention relates to methods for the identification of therapeutic agents for the treatment of osteoporosis and serum lipid lowering agents. The invention relates to isolating cloning, and using nucleic acids from the promoter regions of transforming growth factor β genes comprising novel regulatory elements designated "raloxifene responsive elements". The invention also encompasses eukaryotic cells containing such raloxifene responsive elements operably linked to reporter genes such that the raloxifene responsive elements modulate the transcription of the reporter genes. The invention provides methods for identifying anti-osteoporosis agents that induce transcription of genes operably linked to such raloxifene responsive elements without inducing deleterious or undesirable side effects associated with current anti-osteoporosis therapy regimens.

EP 0 629 697 A3

Jouve, 18, rue Saint-Denis, 75001 PARIS

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EUROPEAN SEARCH REPORT

Application Number
EP 94 30 4432

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,X	JOURNAL OF BIOLOGICAL CHEMISTRY., vol.265, no.31, 1990, BALTIMORE US pages 19128 - 19136 R.L.LAFYATIST ET AL. 'Structural...' * the whole document *	1-4	C12N15/16 C12N15/63 C12Q1/68 A61K31/00
X	CIBA FOUNDATION SYMPOSIA, vol.157, 1991 pages 7 - 28 ROBERTS AB ET AL 'MULTIPLE FORMS OF TGF-BETA - DISTINCT PROMOTERS AND DIFFERENTIAL EXPRESSION'	1,3,4	
A	* abstract *	10	
A	BONE AND MINERAL, vol.7, no.3, 1989 pages 245 - 254 FELDMANN S ET AL 'ANTIESTROGEN AND ANTIANDROGEN ADMINISTRATION REDUCE BONE MASS IN THE RAT' * the whole document *	5,10	TECHNICAL FIELDS SEARCHED (Int.Cl.5)
A	ACTA ONCOLOGICA, vol.31, no.2, 1992, NORWAY pages 143 - 146 KANGAS L. 'Agonistic and antagonistic effects of antiestrogens in different target organs' * the whole document *	10	C07K C12Q
A	WO-A-88 03168 (THE SALK INSITUTE FOR BIOLOGICAL STUDIES) * abstract; claims 1-41 *	5	
A,D	US-A-4 133 814 (C.DAVID JONES ET AL.) * the whole document *	10	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	21 February 1995	Gurdjian, D

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EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT

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A,D	US-A-4 075 227 (C.DAVID JONES ET AL.) * the whole document *	10	
A,D	US-A-4 380 635 (M.K.PATERS) * the whole document *	10	
A	WO-A-88 07579 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) * abstract; claims 1-16 *	5	
A	ENDOCRINOLOGY, 126 (3). 1990. 1449-1456., vol.126, no.3, 1990, BALTIMORE US pages 1449 - 1456 SUNDSTROM S A et al 'THE STIMULATION OF UTERINE COMPLEMENT COMPONENT C3 GENE EXPRESSION BY ANTIESTROGENS'	1-5,10	
P,X, O	CALCIFIED TISSUE INTERNATIONAL, vol.54, no.4, May 1994, BERLIN page 342 Yang N N et al 'Estrogen receptor: One transcription factor, two genomic pathways'	1-4	TECHNICAL FIELDS SEARCHED (Int.Cl.5)
T	JOURNAL OF BONE AND MINERAL RESEARCH, vol.8, no.S1, August 1993 page S118 YANG NN ET AL 'RALOXIFENE, AN ANTIESTROGEN, SIMULATES THE EFFECTS OF ESTROGEN ON INHIBITING BONE-RESORPTION THROUGH REGULATING TGF-BETA-3 EXPRESSION IN BONE' * abstract *	1,10	

The present search report has been drawn up for all claims

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EP 94304432.1

Remark : Although claims 6-9 are directed to a method of treatment of the human/animal body (Article 52 (4)EPC), the search has been carried out and based on the alleged effects of the compound/composition.

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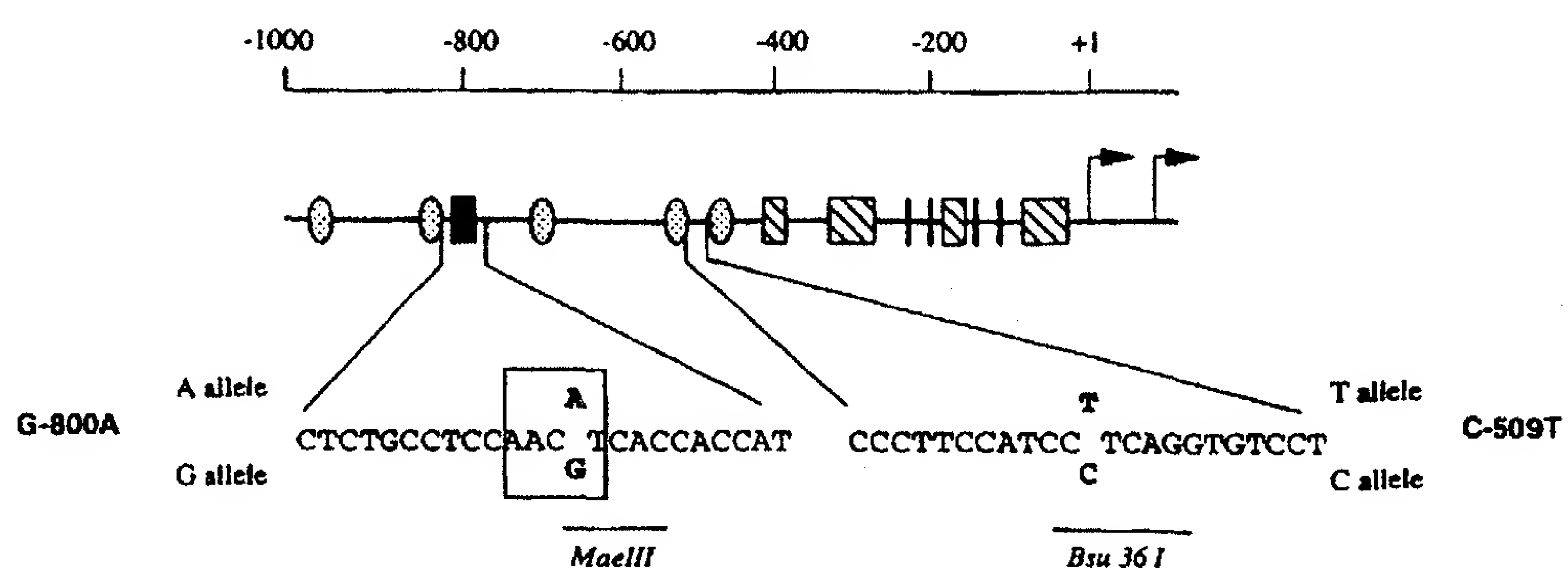
(54) Title: DIAGNOSTIC METHOD AND APPARATUS



(57) Abstract

A method of diagnosis comprises determining genotype of a TGF-β1 promoter - specific polymorphisms that have been identified are at positions-800bp and -509bp. The genotype determined is then used to assess predisposition or susceptibility to a number of diseases: osteoporosis, atherosclerosis, cancer and immune disorders.

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DIAGNOSTIC METHOD AND APPARATUS

This invention relates to diagnostic method and apparatus based upon a polymorphism in a TGF-β gene. More specifically, this invention relates to a method for diagnosis of pre-disposition to certain disease states, by screening for the presence of this polymorphism. The invention also relates to apparatus for screening for the polymorphism. The invention further relates to TGF-β genes containing the polymorphism and to a probe therefor.

A number of major disease states are associated with or correlated with concentration of transforming growth factor β (TGF-β) in circulating plasma. Diseases that have been correlated in this way include atherosclerosis, certain forms of cancer, osteoporosis and a number of auto immune disorders, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, late on set diabetes and others.

In a number of cases, therapy is available for these disease states. However, a problem common to many of these therapies is that whilst the therapy is capable of halting further development of the disease, the therapy is nevertheless not capable of reversing or curing the diseased state to a significant extent.

Hormone replacement therapy is an established treatment for osteoporosis and has proved successful in halting further decline in bone density that is characteristic in women suffering from this disease. Hormone replacement therapy is generally not, however, able to bring about a reversal of osteoporosis, that is to say it is not capable of inducing an increase in the bone density of sufferers.

It would, accordingly, be of particular advantage to be able to identify with increased accuracy those individuals having a predisposition or increased susceptibility to osteoporosis. Suitable therapy could then be put into place before the effects of osteoporosis set in.

A similar situation obtains in respect of atherosclerosis and cancer. In the latter case, treatment of cancer often involves severe side effects. If it were possible to identify individuals with a predisposition or increased susceptibility to cancers then there would be advantage in providing those individuals with preventative therapy to reduce or prevent or delay the onset of cancer as part of a therapy having reduced side effects to those seen with the standard cancer treatments available.

It is an object of this invention to provide method and apparatus for detecting individuals having a predisposition or susceptibility to certain disease states. It is a further object of the invention to identify individuals having such a predisposition or susceptibility by identifying those individuals with an altered TGF-β gene. It is another object of the invention to provide a therapy for those individuals having a predisposition or susceptibility to certain disease states. A still further object of the invention is to provide a therapy for those individuals having a predisposition or susceptibility to certain disease states that are correlated with concentration of TGF-β in circulation.

Accordingly, a first aspect of the invention provides a method of diagnosis comprising determining genotype of a TGF-β1 promoter.

The method of the invention typically comprises determining whether an individual is homozygous or heterozygous for a TGF-β1 promoter and a particular polymorphism thereof. The method is conveniently used to screen for an individual at risk of a condition or disease correlated with TGF-β deficiency, such as osteoporosis, or correlated with elevated levels of TGF-β, such as some cancers.

The DNA sequence of the TGF-β1 promoter region is known and has been published by Kim, S. J. et al, in J. Biol. Chem. 264 (1989) pages 402-408. This sequence is referred to hereafter as the wild type sequence or the published sequence. The method of the invention determines whether the individual being tested has a TGF-β1 promoter which is identical with the published sequence or whether that individual has a TGF-β1 promoter which differs from the published

sequence, i.e. is a polymorphism of the published sequence. In carrying out the invention, an individual's TGF-β1 promoter genotype is generally determined by analysis of a section of the TGF-β1 promoter, rather than by analysis of the entire gene. If the sequence of that section is found to be the same as the corresponding section in the wild type sequence, then that individual is classified as having the wild type TGF-β1 promoter gene.

In use of a specific embodiment of the invention to be described below in further detail, an individual is screened to determine whether he or she possess a TGF-β1 promoter which is the published sequence or is a polymorphism thereof in which a guanine nucleotide at position -800bp has been replaced by an adenine nucleotide. In this specific embodiment, the presence of the polymorphism in which guanine is replaced by adenine at position -800bp correlates with a decreased level of TGF-β in circulation and with a predisposition to certain disease states. As examples of these disease states are included one or more of atherosclerosis, cancers, osteoporosis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and other auto immune disorders.

Screening is carried out, for example, using PCR primers adapted to amplify a portion of the TGF-β1 gene that includes the nucleotide at position -800bp. It is preferred that the PCR primers are selected so as to amplify a region of the gene that surrounds position -800bp and includes at least six nucleotides on either side of this position. PCR techniques are well known in the art and it would be within the ambit of a person of ordinary skill in this art to identify primers for amplifying a suitable section of the TGF-β1 gene including position -800bp. PCR techniques are described for example in EP-A-0200362 and EP-A-0201184.

In a specific embodiment of the invention described in further detail below, the PCR primers have the nucleotide sequences:-

CCCGGCTCCATTTCCAGGTG (SEQ ID NO: 1) and
TGCTCTTGACCACTGTGCCA (SEQ ID NO:2).

The screening is suitably carried out by amplifying a DNA fragment including position -800bp of the TGF-β1 gene and determining whether the amplified region is cleavable by the restriction endonuclease *maeIII*. Cleavage by *maeIII* indicates that the gene is the wild type, while loss of cleavage by *maeIII* indicates that the gene is a variant form, i.e. a polymorphism. It is preferred that the PCR primers are selected so as to be homologous with a region of the genome within 1kb of position -800bp on the TGF-β1 gene.

In a further embodiment of the invention, the diagnostic method comprises analysis of the TGF-β1 promoter using single strand conformational polymorphism (SSCP) mapping. It is preferred that the PCR primers are selected so as to be homologous with a region of the genome within 200bp of position -800bp on the TGF-β1 gene. It is further preferred that the PCR primers are selected so that position -800bp is substantially towards the middle of the amplified DNA segment.

In use of a further specific embodiment of the invention, described in greater detail below, an individual is screened to determine whether he or she possesses a TGF-β1 promoter which is the published sequence or is a polymorphism thereof in which a cytosine nucleotide at position -509bp has been replaced by a thymine nucleotide. In this specific embodiment, the presence of the polymorphism correlates with an elevated level of TGF-β in circulation and with a predisposition to certain disease states, associated with elevated circulating TGF-β, such as some cancers.

Screening may be carried out using PCR primers to amplify a portion of the TGF-β1 gene around position -509. Examples of suitable primers are:

CAGACTCTAGAGACTGTCAG (SEQ ID NO: 3) and
GGTCACCAGAGAAAGAGGAC (SEQ ID NO: 4).

The -509 polymorphism can be detected using Bsu 36I, loss of cleaving indicating presence of the polymorphism.

Alternatively, the -509 polymorphism is detectable using SSCP techniques.

A second aspect of the invention provides diagnostic means comprising PCR primers adapted to amplify a region of a TGF-β1 promoter, preferably a DNA segment comprising a nucleotide at position -800bp, or a segment comprising position -509bp, on the TGF-β1 gene. It is preferred that the PCR primers are adapted to amplify a DNA segment that is up to 2kb in length, more preferably up to 1kb in length. In a particular embodiment of the invention the segment is approximately 400bp in length.

In specific embodiments of the invention described below, the PCR primers are
(i) CCCGGCTCCATTTCCAGGTG (SEQ ID NO:1) and
TGCTCTTGACCACTGTGCCA (SEQ ID NO:2), or
(ii) CAGACTCTAGAGACTGTCAG (SEQ ID NO: 3) and
GGTCACCAGAGAAAGAGGAC (SEQ ID NO: 4).

Optionally, the diagnostic means further comprises means to determine which nucleotide is found at position (a) -800bp or (b) -509bp on the TGF-β1 gene. Examples are the restriction endonuclease *maeIII* for (a) and the restriction endonuclease Bsu 34I for (b). As will be appreciated by a person of skill in the art, an alternative restriction endonuclease that is for example able to cleave the DNA segment when a guanine nucleotide is at position -800bp and is not able to cleave the segment when an adenine nucleotide is at position -800bp is also suitable. Alternatively, a suitable agent would cleave the segment when the nucleotide at position -800bp is adenine and not cleave the segment when this nucleotide is guanine, and likewise for the cytosine/thymine polymorphism at position -509bp. The invention further provides a diagnostic kit comprising diagnostic means according to the second aspect of the invention, optionally within a container.

A third aspect of the invention provides DNA probes comprising a sequence selected from SEQ ID NO:s 1-4.

A fourth aspect of the invention provides a TGF-β1 gene in which a guanine nucleotide at position -800bp is replaced with an adenine nucleotide.

A fifth aspect of the invention provides a TGF-β1 gene in which a cytosine nucleotide at position -509bp is replaced with a thymine nucleotide.

The present invention is based upon the discovery of a single base polymorphism in the TGF-β1 promoter. An aspect of the invention is that the polymorphism is correlated with a predisposition to a number of disease states including, in particular, atherosclerosis, cancers, osteoporosis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and other immune disorders. The invention is of advantage in that by screening for the presence of the polymorphism it is possible to identify individuals likely to have a genetic predisposition to one or more of these disease states.

Accordingly, a sixth aspect of the invention provides a method of therapy comprising screening an individual for a predisposition to osteoporosis and, if a genetic predisposition is identified, treating that individual to delay or reduce or prevent the osteoporosis.

A suitable treatment to prevent or reduce or delay osteoporosis is hormone replacement therapy. The use of this therapy is well known in the art. According to the invention, hormone replacement therapy can thus be commenced in individuals likely to have a predisposition to osteoporosis but in whom osteoporosis has not yet begun to any significant extent.

It is believed that the use of hormone replacement therapy carries with it a concomitant increased risk of breast cancer. The invention offers the advantage that the increased risk of breast cancer associated with hormone replacement therapy can be accepted only by those women who are known to have a likelihood of predisposition to osteoporosis. In an embodiment of the sixth aspect of the invention, the predisposition of an individual to osteoporosis is assessed by

determining whether that individual is homozygous for the wild type TGF-β1 gene, is heterozygous for the wild type and the polymorphism in which guanine at position -800bp is replaced by adenine, or is homozygous for the polymorphism.

According to the invention, an individual who is homozygous for the polymorphism is classified as being at highest risk. An individual being heterozygous is classified as having moderate risk. An individual being homozygous for the wild type TGF-β1 gene is classified as being in the lowest risk category.

Optionally, the assessment of an individual's risk factor is calculated by reference both to the presence of a TGF-β1 promoter polymorphism and also to other known genetic or physiological or dietary or other indications. The invention in this way provides further information on which measurement of an individual's risk can be based.

In a seventh aspect of the invention there is provided a method of therapy in which a predisposition of an individual to atherosclerosis is determined and then, if a predisposition is confirmed, that person is treated to prevent, reduce or delay atherosclerosis. The predisposition to atherosclerosis is assessed, in an embodiment of the invention, using the same criteria as for measurement of predisposition to osteoporosis in the above aspect of the invention. In an embodiment of the seventh aspect, the method comprises determining whether an individual has a predisposition to atherosclerosis and, treating an individual with such a predisposition to prevent, reduce or delay atherosclerosis. For example, the treatment could comprise alterations in that individual's diet. Another treatment could be pharmaceutical, such as by administration of an anticoagulant agent or a fibrinolytic agent. Specific treatments and methods are disclosed in US patents 5242397 and 5171217, the contents of which are incorporated herein by reference.

In further aspects, the invention provides analogous therapies against cancer and auto-immune disorders, based upon identification of a TGF-β1 polymorphism

correlated with a predisposition to these diseases. In the case of cancer, disease may result from either too much TGF-β or too little. Consequently, one such polymorphism is replacement of a cytosine at position -509bp by thymine; another is replacement of a guanine at -800bp by an adenine. In the case of auto-immune disorders, expected to be associated with lowered TGF-β, the polymorphism at -800bp in which adenine replaces guanine would be correlated with predisposition to disease; the polymorphism at -509bp, in which thymine replaces cytosine would be negatively correlated with disease.

The concentration of transforming growth factor β (TGF-β) in circulating plasma has been correlated with the development of several major diseases, including atherosclerosis and certain forms of cancer. However, the mechanisms which control the concentration of TGF-β in plasma are poorly understood. The present invention is based upon our discovery that the concentration of TGF-β in plasma (active TGF-β plus plasma latent TGF-β forms) is predominantly (79%) under genetic control in 66 out of 71 pairs of monozygotic female twins who were post-menopausal. The concentration of active TGF-β was also significantly under genetic control (57%). Analysis of the TGF-β1 promoter by single strand conformational polymorphism (SSCP) mapping has identified a single base polymorphism (A/G at position -800bp) which is significantly associated ($p < 0.01$; $n = 276$) with lower levels of TGF-β in plasma. These data suggest that predisposition to atherosclerosis or various forms of cancer may be linked to particular alleles within the *TGFb1* locus.

TGF-β is a multifunctional cytokine which regulates the proliferation and differentiation of a wide variety of cell types in vitro. Recently, pathological misregulation of the TGF-β system has been implicated in the development of several major diseases, including various forms of cancer, atherosclerosis and fibrotic disease. Moses and colleagues showed that local expression of a constitutively active TGF-β1 transgene prevented the development of mammary carcinoma induced either by transgenic overexpression of TGF-a or by the chemical carcinogen DMBA ((1995) Proc. Natl. Acad. Sci USA 92). Similarly, studies from

the groups of Akhurst and Balmain have demonstrated that low levels of TGF-β1 staining is prognostic for a high risk of malignant conversion of benign tumours in the p53 knockout mouse ((1994) Cancer Res. 54, 5831-5836). More recently, Markowitz et al Science 268, 1336-1338, 1995, have identified somatic mutations of the TGF-β type II signalling receptor, which would be likely to render it non-functional, in human colon cancer biopsies. These data suggest that local decreases in TGF-β activity may be involved in transformation to the malignant phenotype in vivo. By contrast, Arteaga et al. J. Clin. Invest. 92, 2569-2576, 1995, have shown that transfecting a transformed cell line with a construct expressing active TGF-β rendered the cells more tumorigenic in vivo, probably because TGF-β is immunosuppressive and immune surveillance was compromised. Consistent with this observation, elevated levels of plasma TGF-β in patients with malignant prostatic tumours and hepatocellular carcinoma have been reported. While local suppression of TGF-β activity may result in malignant conversion, elevated plasma levels of TGF-β are correlated establishment of the tumour.

In studies on the role of TGF-β in atherogenesis, we have shown that mice expressing the apolipoprotein(a) transgene develop diet-induced lipid lesions resembling early human atherosclerotic plaques at sites in the vessel wall where TGF-β activity is locally depressed by high concentration of apolipoprotein(a). It has also been shown that tamoxifen, which elevates TGF-β activity both in the vessel wall and serum of mice, will prevent diet-induced lipid lesion formation in both apolipoprotein(a) mice and in C57Bl6 inbred mice. Consistent with these observations we have shown that the concentration of active TGF-β is depressed by five fold in individuals with severe coronary atherosclerosis compared to individuals with normal coronary arteries determined by angiography. Taken together, these studies suggest that decreased TGF-β activity, either in the vessel wall or in the circulation, is an important step in the development of atherosclerosis. Despite these correlations with major diseases, the mechanisms which control the concentration of TGF-β in circulating plasma are poorly understood. In the present invention we have examined whether there is genetic regulation of plasma TGF-β concentrations.

TGF-β protein in man is derived from three unlinked genetic loci, TGFb1, TGFb2 and TGFb3 which express three protein isoforms TGF-β1, β2 and β3. Studies using isoform-specific ELISA assays have demonstrated that TGF-β2 is not present in human blood and TGF-β3 has been detected in platelet-poor plasma and serum from only 2/22 individuals tested (unpublished observations of the inventors). Most or all of the TGF-β present in blood from most individuals is therefore the b1 isoform. Recent studies have shown that TGF-β1 in blood is present in several different protein complexes. The highest concentrations of TGF-β1 are contained in the platelets, where it is present as two distinct platelet latent complexes, termed large and small latent complexes, which have no known biological activity. In contrast, platelet-poor plasma contains a biologically active form of TGF-β1 and a latent complex, which is distinct from either of the platelet latent complexes, that we have termed plasma latent complex. The same forms of plasma TGF-β are present in serum, together with much larger concentrations of platelet large latent complex which is released into serum when clotting occurs. For our present studies of the genetic control of plasma TGF-β in populations of twins, an ELISA was used which detects only the two forms of plasma TGF-β described above: the active form together with the plasma latent complex. The capture antibody (BDA19; R&D Systems) does not recognise the platelet large latent complex which is released into serum and consequently the BDA19 ELISA detects the same concentrations of TGF-β in plasma or serum prepared from the same blood sample.

There now follows description of specific embodiments of the invention illustrated by figures 1-4 in which:-

Fig 1 shows the circulating concentration of TGF-β in twin pairs. The concentration of active plus plasma latent TGF-β (a,b) and active TGF-β (c,d) was measured in serum from monozygotic (a,c) and dizygotic (b,d) twins. All individuals were female, post-menopausal and not receiving hormone replacement therapy. For each pair, twin 1 was arbitrarily designated as the sibling with lowest circulating concentration of TGF-β.

Fig 2 shows a polymorphism in the TGF-β1 promoter associated with circulating TGF-β concentration. (a) Single strand conformation polymorphism (SSCP) acrylamide gel of non-denatured DNA obtained by polymerase chain reaction between primers 5'-CCCGGCTCCATTTCCAGGTG-3' (-1106 to -1125bp) (SEQ ID NO: 1) and 5'-TGCTCTTGACCACTGTGCCA-3' (-738p to -757bp) (SEQ ID NO: 2) with lymphocyte-derived genomic DNA from four healthy Caucasian donors as the template. One heterozygote (lane 1) is shown with the diagnostic doublet indicated by an arrow. (b) Diagrammatic representation of the putative transcription factor binding sites in the TGFb1 promoter. Hatched box : one or more consensus sp1 binding sites; vertical line : consensus ap2 binding site; stippled oval : consensus glucocorticoid response element; filled box : consensus CREB half-site. A portion of the wild type sequence is shown - CTCTGCCTCCAACGTCACCACCAT = SEQ ID NO: 5. The sequence of the CREB half-site is shown (boxed) with the single base polymorphism that resulted in the SSCP doublet shown in (a) marked in bold script. The maeIII consensus sequence present in the G allele (GTNAC) is underlined. All nucleotide positions are related to the most 5' transcriptional start site of the TGFb1 gene described in J Biol Chem, 264, pages 402-408, 1989.
(c) Distribution of circulating concentration of TGF-β in those twin pairs analyzed in Fig. 1 who have no A allele identified by the absence of undigested DNA in the maeIII digestion of the PCR fragment obtained in (a) above. (d) Distribution of the circulating concentration of TGF-β in individuals with AG genotype.

Fig. 3 shows the location of polymorphisms in the TGFb1 promoter, and includes a diagrammatic representation of the putative transcription factor binding sites in the TGFb1 promoter. Hatched box : one or more consensus sp1 binding sites; vertical line : consensus ap2 binding site; stippled oval : consensus nuclear hormone binding element; filled box : consensus CREB half-site. Portions of the wild type sequence are shown - CTCTGCCTCCAACGTCACCACCAT = SEQ ID NO: 5, CCCTTCCATCCCTCAGGTGTCCT = SEQ ID NO: 6. The sequence of the CREB half-site is shown (boxed) with the single base polymorphism at -800bp marked in bold script. The maeIII consensus sequence present in the G allele (GTNAC) is underlined. The sequence surrounding the single base polymorphism at -509bp

(bold script) is also shown. All nucleotide positions are related to the most 5′ transcriptional start site of the TGFb1 gene.

Fig 4 shows association between polymorphisms in the TGFb1 promoter region and circulation concentration of TGF-β Boxplots (inter-quartile range) of (a+l) (a,c) TGF-β concentrations and active (b,c) TGF-β concentrations in groups of subjects sorted by genotype at -800bp (a,b) and -509bp (c,d).

Example 1

TGF-β was assayed by BDA19 ELISA in serum samples from 136 pairs of twins (71 monozygotic and 65 dizygotic pairs) to assess the genetic contribution to variation in TGF-β concentration. The concordance in concentration between the monozygotic siblings was very high (sn/mean = 16.9% +/- 2.3 %; n=66) in 66/71 pairs. For each of the remaining five pairs, one sibling had an elevated level of circulating TGF-β which was higher than in any of the other 66 twin pairs and was more than five standard deviations from mean of the population as a whole. It is very likely that these individuals had elevated concentrations of circulating TGF-β due to environmental influences and these five twin pairs were excluded from further analysis. For the remaining monozygotic twin pairs, the intraclass correlation coefficient (r^2MZ) was 0.79 (Fig 1a).

Using the same exclusion criteria for the dizygotic twins, there were two twin pairs in which one sibling had a concentration of TGF-β greater than for any of the concordant monozygotic twin pairs and more than five standard deviations from the mean of the whole population. For the remaining 63 dizygotic pairs the correlation was less marked (intraclass correlation coefficient r^2D2=0.39; Fig 1b) than for the monozygotic twin pairs. If genetic variation at a single locus were responsible for the variation in TGF-β concentration, the r^2DZ to r^2MZ ratio would be predicted to be 0.5 and as the number of unlinked alleles contributing to the control of TGF-β concentration increases, the r^2DZ to r^2MZ ratio decreases. In this study r^2MZ / r^2DZ =0.49, suggesting that genetic variability at very few unlinked loci, or possibly at

only one locus, is responsible for the genetic control of circulating TGF-β.

The concentrations of active TGF-β in the serum samples were also measured using an ELISA in which the extracellular domain of the type II TGF-β receptor (R2X) is the capture agent. The concentration of active TGF-β in the circulation was also significantly correlated for the monozygotic twin pairs (r^2MZ =0.57; Fig 1c), but much less so in the dizygotic twins (r^2DZ = 0.11; Fig 1d). Since the r^2DZ to r^2MZ ratio is much lower than 0.5 we conclude that a number of unlinked genetic loci are likely to contribute to the variation in the concentration of active TGF-β. This is consistent with our previous observation that activation of TGF-β was inversely correlated with the concentration of lipoprotein(a), since the circulating concentration of Lp(a) is genetically determined at a locus unlinked to TGFb1.

Since most of the TGF-β in plasma is the b1 isoform we investigated whether mutations at the TGFb1 locus influence the circulating concentration of TGF-β. Using SSCP mapping a polymorphism was identified in the promoter region of the TGFb1 gene approximately 1kb upstream from the transcriptional start site (Fig 2a,b). Sequencing of the PCR fragment analyzed by SSCP mapping identified the polymorphism as a single base change at position -800bp, with A replacing the G in the published genomic sequence ((1989) J. Biol. Chem. 264, 402-408) in approximately 10% of alleles. The presence of adenine at position -800bp destroyed a restriction site for the endonuclease maeIII which was therefore used to scan the promoter from the 136 twin pairs for the presence of this polymorphism. 52/276 individuals were heterozygous for this polymorphism and one monozygotic twin pair (2 individuals out of 276) were homozygous for A at position -800bp. The polymorphism was therefore present in this population at a frequency of 9.8% of alleles tested.

We tested whether the individuals with rare A allele had circulating levels of TGF-β significantly different from the more common G allele. The mean TGF-β serum concentration assayed by BDA19 ELISA in individuals with GG genotype was 6.4 +/- 0.6 ng/ml (n=252; Fig 2c), not significantly different from the mean TGF-β

concentration of 8.9 +/- 1.5 reported for a group of female donors of similar mean age. By contrast, the mean TGF-β concentration in individuals with AG genotype was 4.2 +/- 1.1 ng/ml ($p<0.01$; $n=62$; Fig 2d). The siblings of the monozygotic twin pair homozygous for the A allele had TGF-β concentration of < 1ng/ml (below the detection limit of the assay used) and 2ng/ml. Kruskal-Walis test for difference between GG and AG genotypes is $P=0.01$ taking all twins into account as independent individuals. We conclude that the presence of the A allele is significantly associated with lower circulating concentrations of TGF-β.

The polymorphism described is present in a consensus CREB half-site and the A allele would be expected to have reduced affinity for the CREB family of transcription factors. The polymorphism described may therefore be directly responsible for the different concentrations of TGF-β or alternatively it may be in linkage disequilibrium with other as yet unidentified polymorphisms in the TGFb1 locus which are affecting the concentration of TGF-β. Taken together, the data suggest that a major part of the genetic control over circulating levels of TGF-β may reside in the TGFb1 locus, since only a very few unlinked loci, or possibly only a single locus, are implicated in the control mechanism and we have identified a polymorphism in the TGFb1 locus which is correlated with TGF-β concentration.

Example 2

The present Example follows on from the work reported in the first Example above.

Subjects

All the subjects tested here were female (age range 39 to 70 years; mean 57.7 years) and the majority were post-menopausal. Twins were recruited following a national media campaign and were broadly representative of the normal United Kingdom population. None of the subjects were taking hormone replacement therapy or other hormonally active medications. Serum was prepared and stored for TGF-β analysis. Lymphocyte DNA was also prepared for each subject using the standard phenol extraction method and zygosity status was determined by questionnaire and multiplex fingerprinting.

TGF-*β* analysis

Active plus acid-activatable latent (a+l) TGF-*β* was measured using the BDA19 capture ELISA. Active TGF-*β* was measured using the R2X capture ELISA. A single determination of each sample was made. The intra-assay co-efficient of variation of the assay used are 6.8% and 7.4% respectively. Serum samples with TGF-*β* concentration below the detection limit of the assays used (<1ng/ml; 27/334 for (a+l) TGF-*β* and 37/334 for active TGF-*β*) were assigned a value of 0.5ng/ml.

SSCP

Polymerase chain reaction (PCR) was used to amplify fragments of the promoter that were approximately 400bp long. 1.0mg of genomic DNA was added to a 20ml reaction consisting of 1x Taq Polymerase Buffer (Pharmacia), 50nmol of each dNTP, and 6.25pmol forward primer. The reaction was heated to 95 degrees C for 30s and then held at 80 degrees C while 5ml containing 6.25pmol reverse primer and 0.5 units Taq Polymerase in 1x Taq Polymerase Buffer was added. Samples were amplified for 35 cycles of 95 ITCH for 1 minute, 60 ITCH for 1 minute and 72 ITCH for 1 minute, followed by an extension period of 10 minutes at 72 ITCH. Primers were 5'-CCCGGCTCCATTTCCAGGTG-3'(SEQ ID NO: 1) and 5'-TGCTCTTGACCACTGTGCCA-3'(SEQ ID NO: 2) for G-800A polymorphism and 5'-CAGACTCTAGAGACTGTCAG-3'(SEQ ID NO: 3) and 5'-GGTCACCAGAGAAAGAGGAC-3'(SEQ ID NO: 4) for C-509T polymorphism. PCR products were denatured with 0.2M sodium hydroxide and 0.5mM disodium ethylenediaminetetraacetic acid at room temperature. Single stranded DNA bands were resolved on a 20% acrylamide gel (Phastsystem, Pharmacia or XCell II system, R&D Systems) and visualised by silver staining.

Genotyping

G-800A polymorphism abolishes a Mae III restriction site. Mae III and Mae III Buffer (Boehringer) were added directly to the PCR products to a final volume of 30 ml, and incubated at 55 ITCH for 5 hours. Digests were resolved on a 1.2% agarose gel. C-509T polymorphism is in a Bsu 36 I restriction site. PCR products were precipitated with 3 volumes 96% ethanol and 1/10 volume 3 M Sodium Acetate pH 5.2 at -20

ITCH for 1 hour, followed by centrifugation at 13,000 r.p.m. for 10 mins. The DNA was resuspended in a 20 ml Bsu 36 I digest containing 10 units Bsu 36 I (New England Biolabs) and incubated at 37 ITCH for a minimum of 12 hours. Digests were resolved by 1.5% agarose gel electrophoresis.

TGF-β was assayed by BDA19 ELISA in serum samples from 174 pairs of twins (87 monozygotic (MZ) and 87 dizygotic (DZ) pairs) to assess the genetic contribution to variation in TGF-β concentration. The baseline characteristics of these twins are shown in Table 1. The MZ twins were slightly older than the DZ twins (mean age 58.9 years versus 56.6 years) and a significantly higher proportion were post-menopausal (87% versus 75%). However, neither of these variables were significantly associated with the concentration of (a+l) or active TGF-β. Consistent with our previous studies, the plasma (a+l) TGF-β concentrations of this population did not conform to a normal distribution. A boxcox transformation was applied to maximise normality, which resulted in equal means and variances between the MZ and DZ groups but the distributions remained significantly non-normal. We therefore treated TGF-β concentration as a categorical variable, assigning each individual into an approximate tertile (for (a+l) TGF-β these were 'low', less than 4ng/ml; 'middle' 4-5ng/ml; 'high' more than 5ng/ml). For (a+l) TGF-β, the percent concordance (that is the proportion of twin pairs where both individuals fell into the same tertile) for MZ pairs (57.8%) was significantly higher than for DZ pairs (40.5%; p = 0.025; Chi-square test; Table 2). This demonstrates that the concentration of (a+l) TGF-β in plasma is influenced by genetic factors.

The concentrations of active TGF-β in the serum samples were also measured using an ELISA in which the extracellular domain of the type II TGF-β receptor (R2X) as the capture agent. The distribution of active TGF-β concentrations for both groups of twins were significantly different from a normal distribution, even after boxcox transformation, as for the distribution of (a+l) TGF-β concentrations. Treating the active TGF-β concentration as a categorical variable ('low' less than 3ng/ml; 'middle' 3 or 4ng/ml; 'high' greater than 4ng/ml), the percent concordance between tertiles was again significantly higher for MZ twins (62.8%) than for DZ twins (43.6%; p =

0.016; Chi-square test; Table 2). It appears, therefore, that the concentration of active TGF-β in plasma is also under genetic control.

Since most of the TGF-β in plasma is the TGF-β1 isoform we investigated whether mutations at the TGFb1 locus influence the circulating concentration of TGF-β. Using SSCP analysis two polymorphisms were identified in the promoter region of the TGFb1 gene within 1.5kbp upstream from the major transcriptional start site. Sequencing of the PCR fragments analyzed by SSCP mapping identified two single base substitution polymorphisms. The first polymorphism occurred at -800bp, with adenine replacing guanine in the published genomic sequence in approximately 10% of alleles (G-800A; Fig 3). The second polymorphism was at -509bp with thymine replacing cytosine in approximately 30% of alleles (C-509T; Fig 3). The genotype at these two sites in the TGFb1 promoter region was determined for the majority of the individuals in the twin study. For the G-800A polymorphism we found 59/341 individuals were heterozygous (AG) and two individuals (one monozygotic twin pair) were homozygous AA, corresponding to an A allele frequency of 0.092 in this population. For the C-509T, 160/330 individuals were heterozygous (CT), and 24/330 individuals were homozygous for thymine at this position (T allele frequency = 0.315). The G-800A polymorphism is in Hardy-Weinberg equilibrium within the twin study (c2=0.345, p =0.557), however the C-509T polymorphism does not exhibit Hardy-Weinberg equilibrium when all twins are included (c2=5.012, p=0.025).

We tested whether there was any association between the TGFb1 promoter genotype and plasma concentration of (a+l) TGF-β. The presence of an A allele is significantly associated with lower circulating concentrations of TGF-β, irrespective of whether all individuals are included (tied p = 0.0005; n =328; Kruskal-Wallis test) or if only one individual selected at random is included from each twin pair (tied p = 0.02; n=164; Kruskal-Wallis test; Fig 4a). The presence of the T allele at -509bp is significantly associated with higher plasma concentrations of TGF-β (tied p = 0.0004; n=319; Kruskal-Wallis test), and this association is still significant if only one twin from each pair is included in the analysis (tied p=0.016, Kruskal-Wallis test; Fig 4c). The G-800A and C-509T mutations are in repulsion, as genotypes such as AGTT, which

would require adenine and thymine to be in linkage have never been observed.

It was observed that (Fig 4b,d) the T allele at -509bp is significantly associated with higher concentrations of active TGF-*β* (p=0.023; Kruskal-Wallis test with only one twin from each pair included) and the A allele -800bp is likely to be asspciated with lower concentrations of active TGF-*β* (p=0.076; Kruskal-Wallis test with only one twin from each pair included).

The presence of polymorphisms in the TGFb1 locus may indicate predisposition to diseases that have been linked to circulating levels of TGF-*β*, including atherosclerosis (decreased circulating TGF-*β*1) and some forms of cancer (elevated circulating TGF-*β*1).

The invention thus provides method and apparatus for identification and treatment of individuals having a TGF-*β*1 polymorphism, correlated with a predisposition to certain disease states.

TABLE 1

	MZ (n=174)	DZ (n=174)	p
Age (years)	58.9 ± 6.6	56.6 ± 8.3	0.006
Height (cm)	160.2 ± 6.1	161.3 ± 5.9	0.078
Weight (kg)	62.9 ± 9.7	65.0 ± 11.0	0.063
No. (%) post-menopausal	131 (87)	112 (75)	0.007
No. (%) current smoker	24 (14)	33 (19)	0.325
No. (%) on no alcohol	23 (13)	15 (9)	0.121
No. (%) previous HRT use	32 (18)	31 (18)	0.889
No. (%) hysterectomy	35 (20)	37 (21)	0.791

Baseline characteristics of population. Only age and the number of post-menopausal individuals differed between the monozygous (MZ) and dizygous (DZ) groups (p values highlighted in bold). None of the individuals studied were currently on hormone-replacement therapy or taking other hormonally active medications. Abbreviation : HRT, hormone replacement therapy.

TABLE 2

(a) (a+l) TGF-β

			TWIN 2 'Low'	TWIN 2 'Middle'	TWIN 2 'High'
TWIN 1	'Low'	MZ	12%	12%	4%
		DZ	11%	14%	5%
	'Middle'	MZ	4%	17%	17%
		DZ	11%	17%	13%
	'High'	MZ	2%	4%	29%
		DZ	6%	11%	13%

(b) active TGF-β

			TWIN 2 'Low'	TWIN 2 'Middle'	TWIN 2 'High'
TWIN 1	'Low'	MZ	12%	5%	4%
		DZ	18%	14%	3%
	'Middle'	MZ	6%	30%	14%
		DZ	8%	18%	12%
	'High'	MZ	3%	5%	22%
		DZ	8%	12%	8%

Within-pair concordance of (a+l) and active TGF-β concentrations between MZ and DZ twin pairs. The concentration of (a+l) (a) or active (b) TGF-β was treated as a categorial variable, and individuals assigned to approximate tertiles (for (a+l) TGF-β : 'low', <4ng/ml; 'middle' 4-5ng/ml; 'high' >5ng/ml and for active TGF-β : 'low', <3ng/ml; 'middle' 3-4ng/ml; 'high' >4ng/ml). The percentage of twin pairs in each of the MZ and DZ groups are shown separately for each combination of TGF-β concentration categories. The percentage pairwise concordance for each group is the proportion of twin pairs on the diagonal of each table. The percentage pairwise concordance is significantly higher from MZ than DZ twins for both (a+l) TGF-β (p=0.025; Chi-square test) and active TGF-β (p=0.016; Chi-square test). Where the proportion of twins in the cell differs significantly between the MZ and DZ groups (p<0.05; Chi-square test) the percentages are highlighted in bold.

CLAIMS

1. A method of diagnosis comprising determining genotype of a TGF-β1 promoter.

2. A method of diagnosis according to Claim 1 comprising determining whether an individual possesses a wild type TGF-β1 promoter.

3. A method of diagnosis according to Claim 1 or 2 comprising determining whether an individual possesses a wild type TGF-β1 promoter or a variant promoter that differs from the wild type by at least a single nucleotide substitution.

4. A method according to claim 3 wherein the variant differs from the wild type in that cytosine at position -509bp is substituted by thymine.

5. A method according to Claim 3 wherein the variant differs from the wild type in that guanine at position -800bp is substituted by adenine.

6. A method according to any of Claims 1-5 comprising amplifying a portion of that individual's TGF-β1 gene using PCR techniques.

7. A method according to any preceding claims for diagnosis of a disease selected from osteoporosis, atherosclerosis, cancer and immune disorders.

8. Diagnostic means, comprising means for determining genotype of a TGF-β1 promoter.

9. Diagnostic means according to Claim 8, comprising PCR primers adapted to amplify a region of a TGF-β1 promoter.

10. Diagnostic means according to Claim 9 comprising PCR primers adapted to

amplify a DNA segment comprising a nucleotide at position -800bp on a TGF-β1 gene.

11. Diagnostic means according to Claim 9 comprising PCR primers adapted to amplify a DNA segment comprising a nucleotide at position 509bp on a TGF-β1 gene.

12. Diagnostic means according to Claim 9 wherein the PCR primers are selected from (i) SEQ ID NO: 1 and SEQ ID NO: 2 and (ii) SEQ ID NO: 3 and SEQ ID NO: 4.

13. Diagnostic means according to any of Claims 8-12 further comprising an enzyme such as a restriction endonuclease capable of cleaving a wild type TGF-β1 promoter at position -800bp and not capable of cleaving a variant of the wild type promoter in which the nucleotide at position -800bp is adenine.

14. Diagnostic means according to any of Claims 8-12 further comprising an enzyme such as a restriction endonuclease capable of cleaving a wild type TGF-β1 promoter at position -509bp and not capable of cleaving a variant of the wild type promoter in which the nucleotide at position -509bp is thymine.

15. DNA comprising a TGF-β1 gene, or a fragment thereof at least 15 nucleotides in length, in which guanine at position -800bp is substituted by adenine, or DNA complementary thereto.

16. DNA comprising a TGF-β1 gene, or a fragment thereof at least 15 nucleotides in length, in which cytosine at position -509bp is substituted by thymine, or DNA complementary thereto.

17. A method of osteoporosis therapy comprising:-

screening an individual for a genetic predisposition to osteoporosis; and

if such a predisposition is identified, treating that individual to prevent or reduce osteoporosis or to delay the onset of osteoporosis.

18. A method according to Claim 17 wherein a predisposition to osteoporosis is correlated with a TGF-β1 promoter gene which varies from the wild type promoter sequence.

19. A method according to Claim 17 or 18 in which a predisposition to osteoporosis is correlated with a TGF-β1 promoter in which at position -800bp a guanine nucleotide is substituted by an adenine nucleotide.

20. A method according to any of Claims 17-19 comprising treating the individual by hormone replacement therapy.

21. A method of atherosclerosis therapy comprising:-

screening an individual for a genetic predisposition to atherosclerosis; and

if such a predisposition is identified, treating that individual to prevent or reduce atherosclerosis or to delay the onset of atherosclerosis.

22. A method according to Claim 21 wherein a predisposition to atherosclerosis is correlated with a TGF-β1 promoter gene which varies from the wild type promoter sequence.

23. A method of cancer therapy comprising:-

screening an individual for a genetic predisposition to cancer; and

if such a predisposition is identified, treating that individual to prevent or reduce cancer or to delay the onset of cancer.

24. A method according to Claim 23 wherein a predisposition to cancer is correlated with a TGF-β1 promoter gene which varies from the wild type promoter sequence.

25. A method according to Claim 23 wherein a predisposition to cancer is correlated with a TGF-β1 promoter gene in which cytosine at position -509bp is substituted by thymine.

26. A method according to Claim 23 in which a predisposition to cancer is correlated with a TGF-β1 promoter in which at position -800bp a guanine nucleotide is substituted by an adenine nucleotide.

27. A method of immune disorder therapy comprising:-

    screening an individual for a genetic predisposition to immune disorder; and

    if such a predisposition is identified, treating that individual to prevent or reduce immune disorder or to delay the onset of immune disorder.

28. A method according to Claim 27 wherein a predisposition to immune disorder is correlated with a TGF-β1 promoter gene in which cytosine at position -509bp is substituted by thymine.

29. A method according to Claim 27 in which a predisposition to immune disorder is correlated with a TGF-β1 promoter in which at position -800bp a guanine nucleotide is substituted by an adenine nucleotide.

30. A diagnostic kit comprising means according to any of claims 8-14.

31. Use, in the manufacture of means for assessing whether an individual has a predisposition to osteoporosis, of PCR primers adapted to amplify a region of a TGF-β1 promoter.

32. Use, in the manufacture of means for assessing whether an individual has a predisposition to cancer, of PCR primers adapted to amplify a region of a TGF-β1 promoter.

33. Use, in the manufacture of means for assessing whether an individual has a predisposition to atherosclerosis, of PCR primers adapted to amplify a region of a TGF-β1 promoter.

34. Use, in the manufacture of means for assessing whether an individual has a predisposition to immune disorder, of PCR primers adapted to amplify a region of a TGF-β1 promoter.

35. A method of diagnosis of predisposition to osteoporosis comprising determining a genetic disposition to osteoporosis.

36. A method of diagnosis of predisposition to cancer comprising determining a genetic disposition to cancer.

37. A method of diagnosis of predisposition to atherosclerosis comprising determining a genetic disposition to atherosclerosis.

38. A method of diagnosis of predisposition to immune disorder comprising determining a genetic disposition to immune disorder.

38. A probe for identification of a polymorphism of a TGF-β1 promoter comprising a section of DNA of the promoter in which guanine at position -800bp has been replaced by adenine, or cytosine at position -509bp has been replaced by thymine, or a section of DNA complementary thereto.

39. A probe according to claim 38 being at least 15 nucleotides in length.

1/4

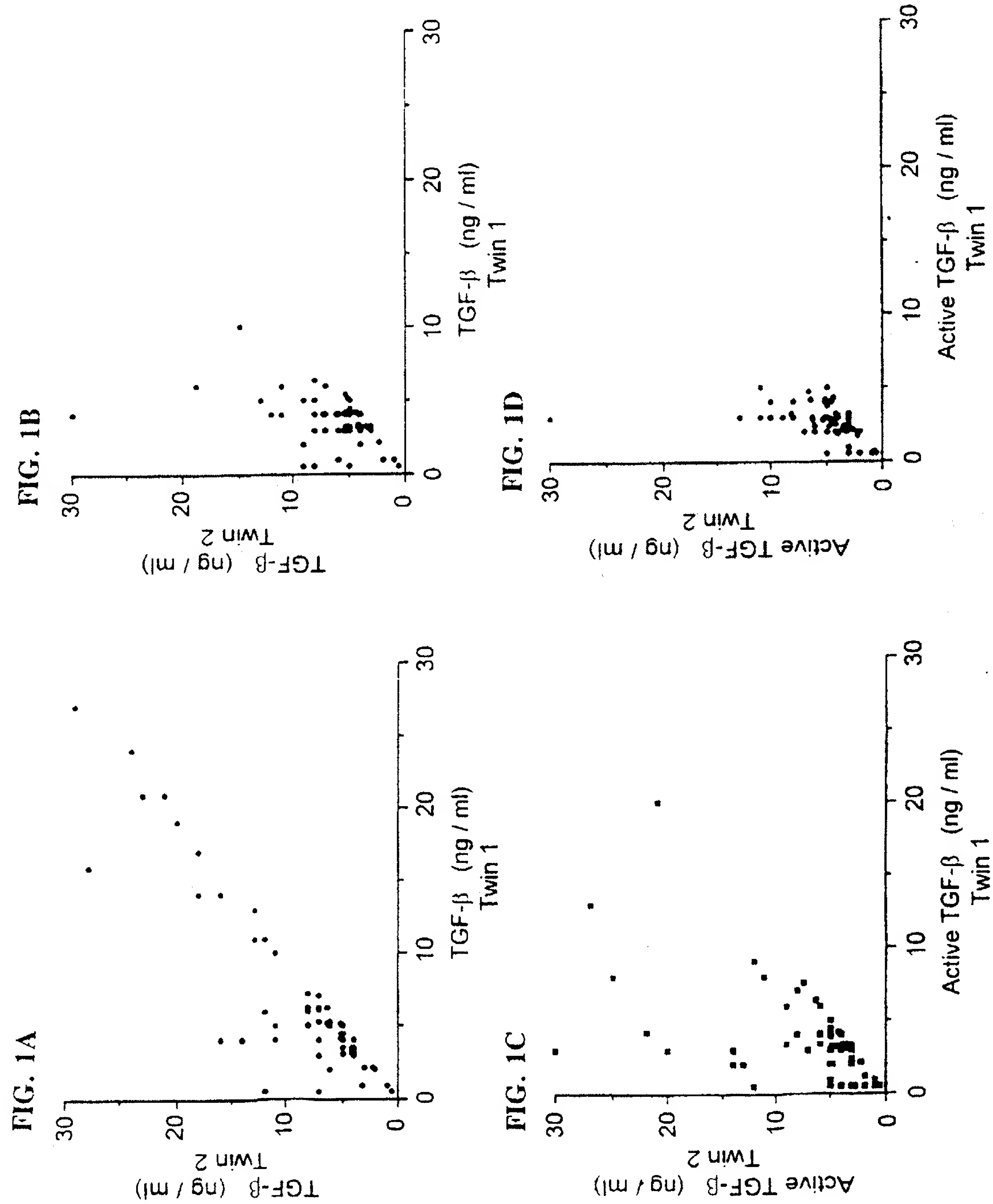


FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 2A

FIG. 2B

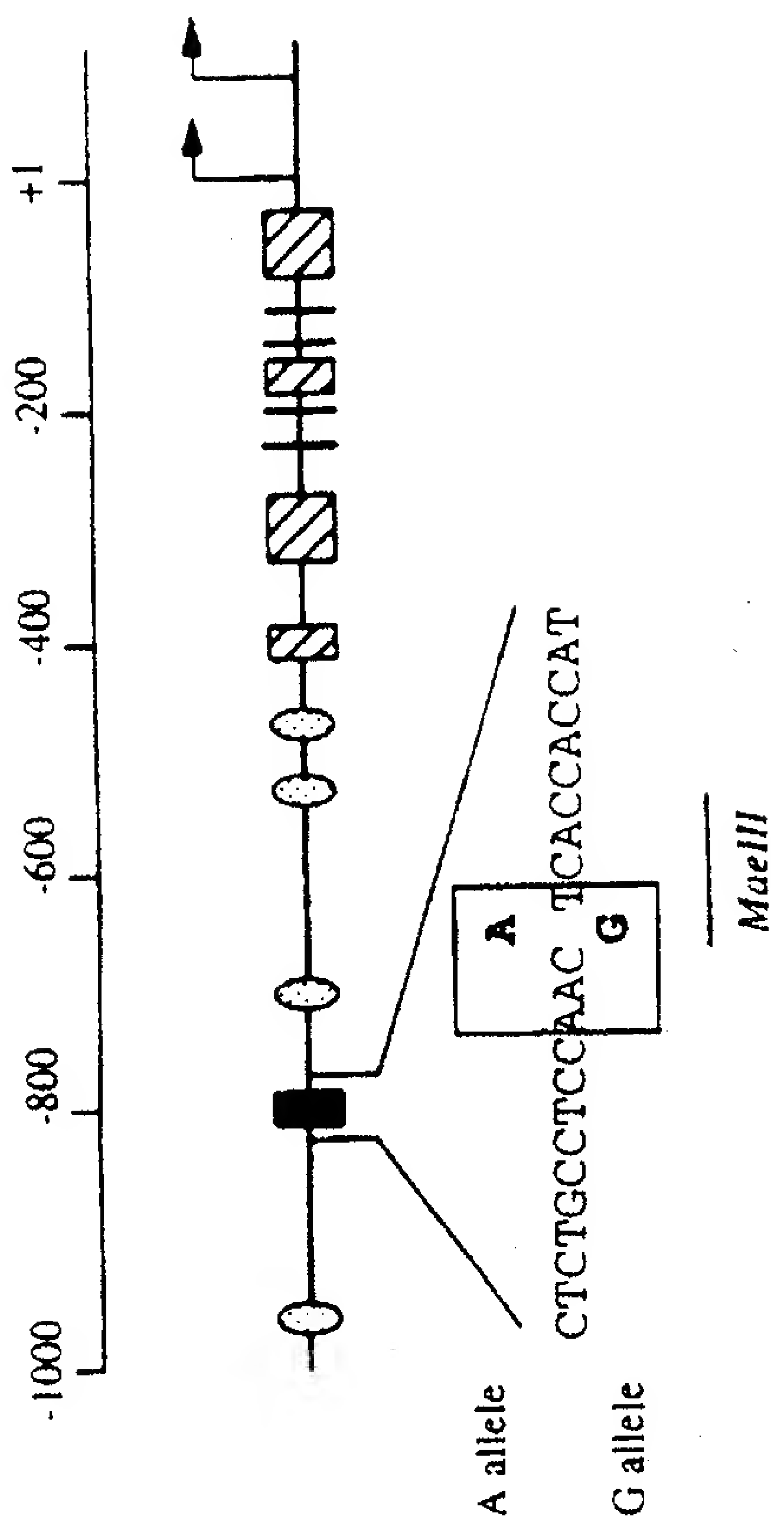


FIG. 2C

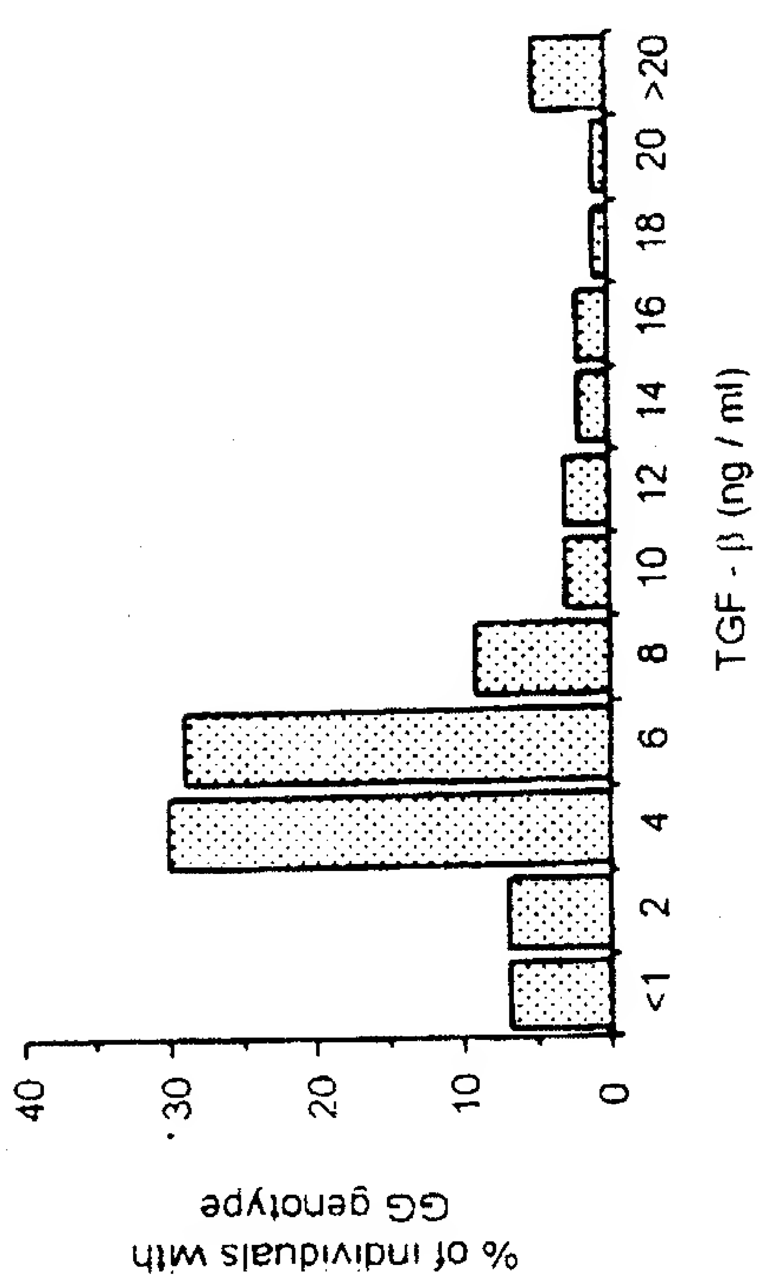
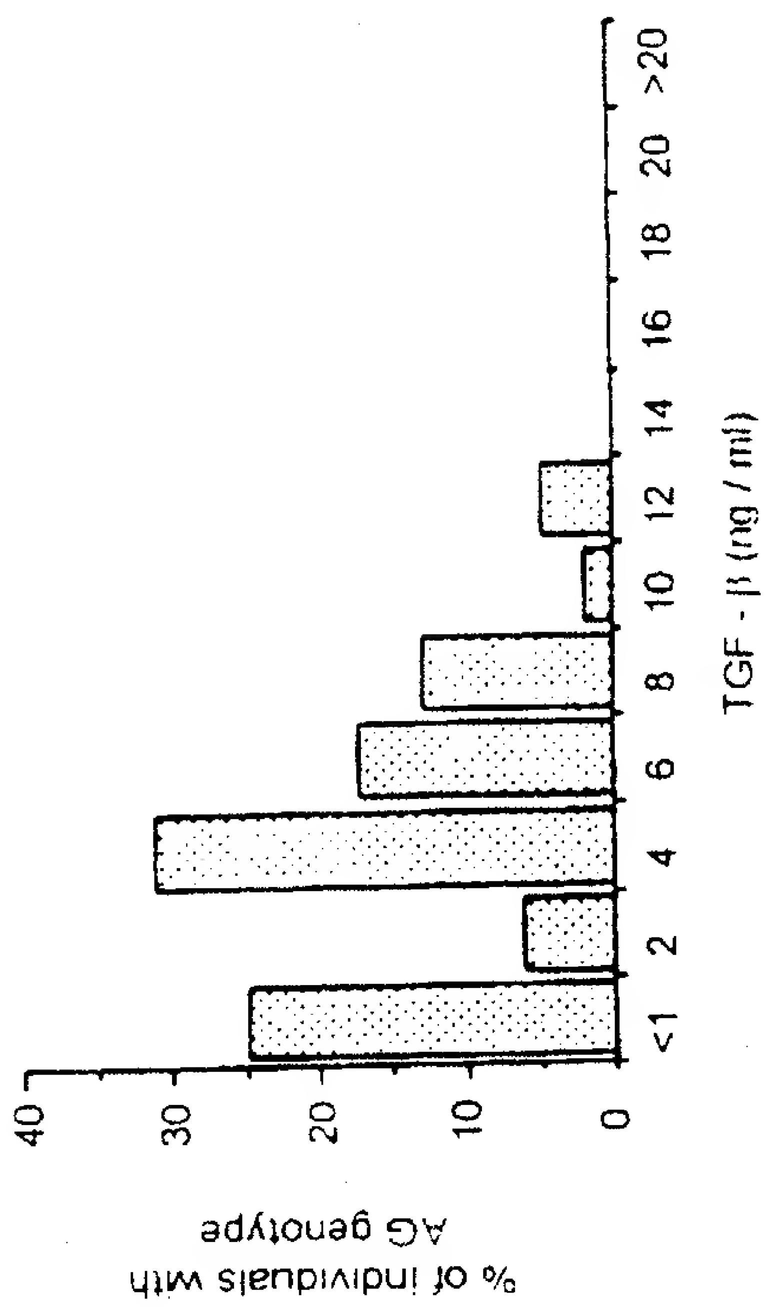


FIG. 2D



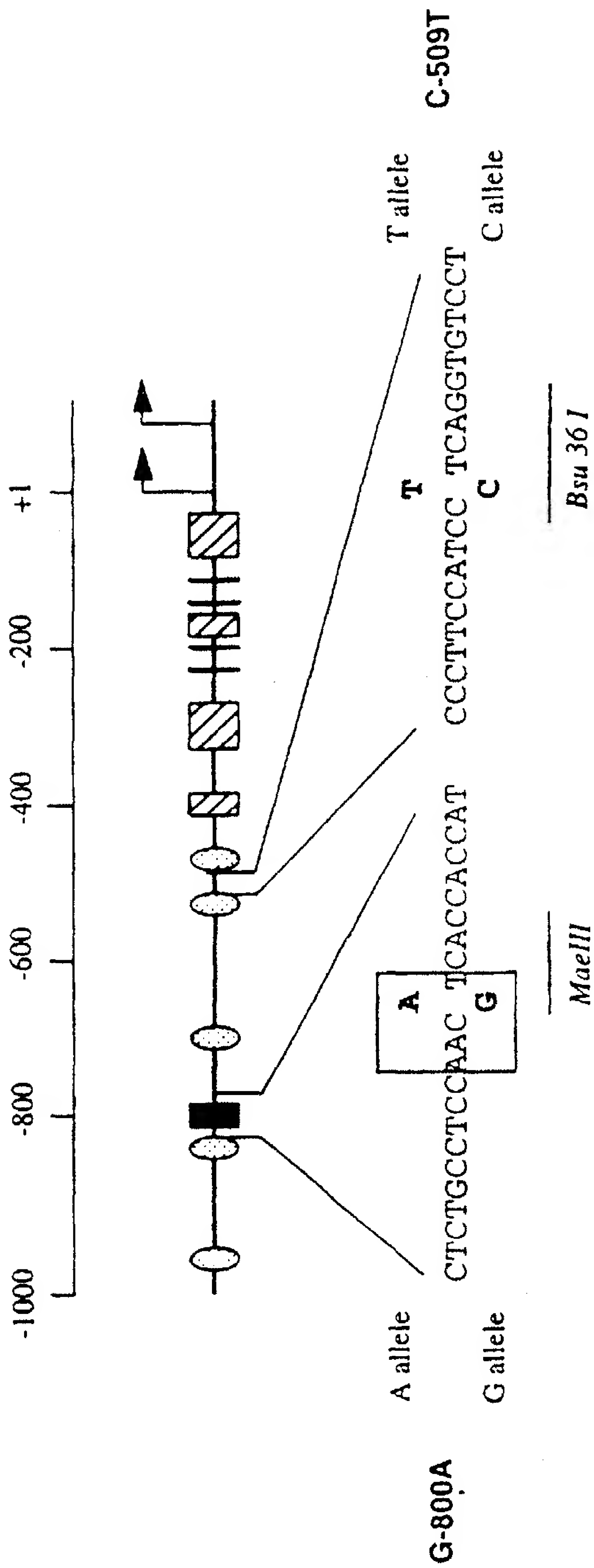

C-509T
T allele
C allele
CCCTTCCATCC TCAGGTGTCCT
T
C
Bsu 36 I
CTCTGCCTCCAAC TCACCACCAT
A
G
MaeIII
A allele
G allele
G-800A
-1000
-800
-600
-400
-200
+1

FIG. 3

4/4

FIG. 4A

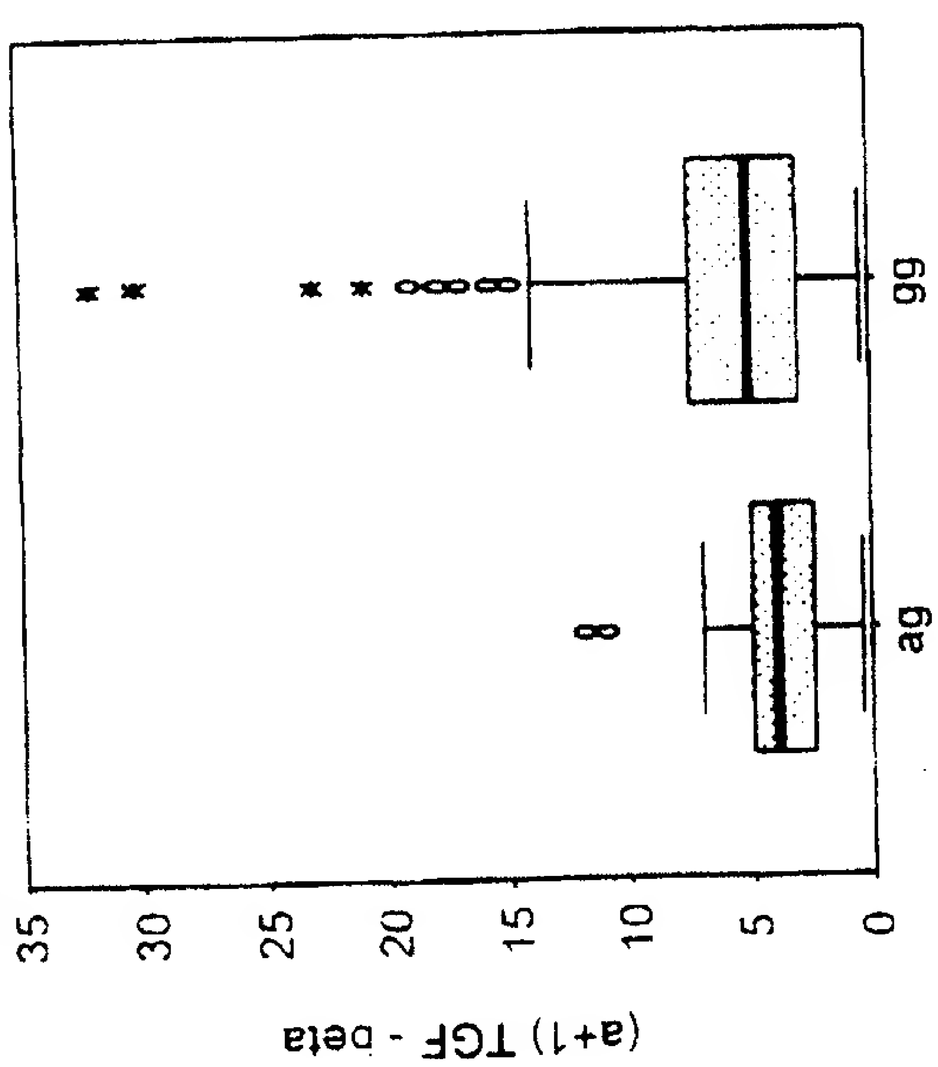


FIG. 4B

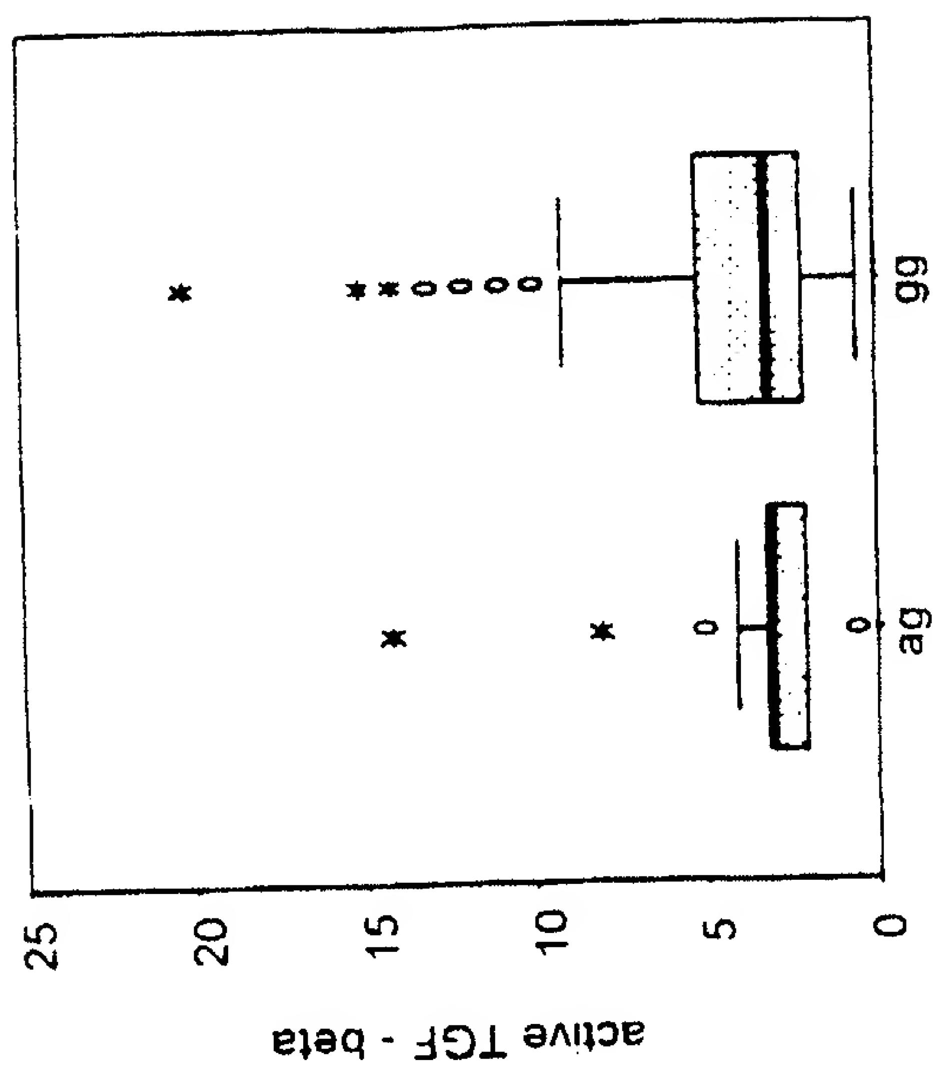


FIG. 4C

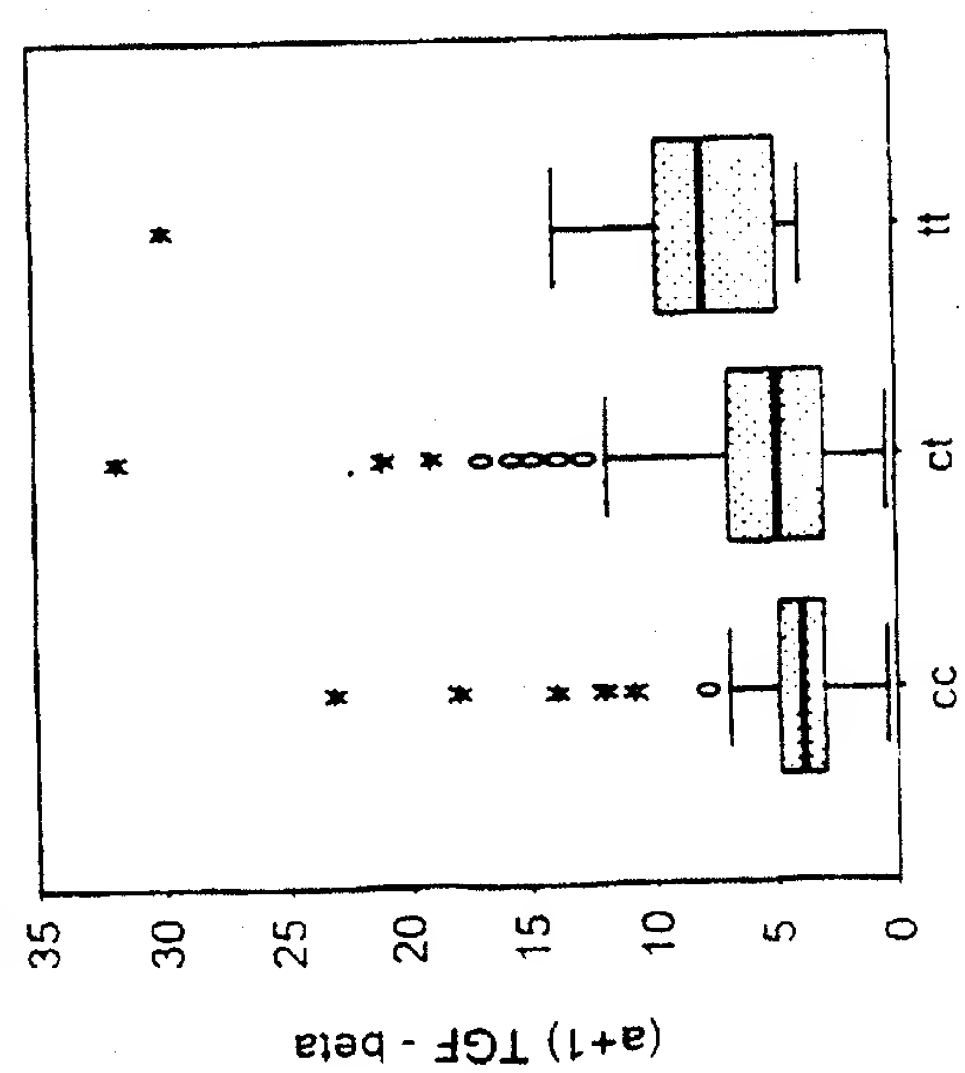
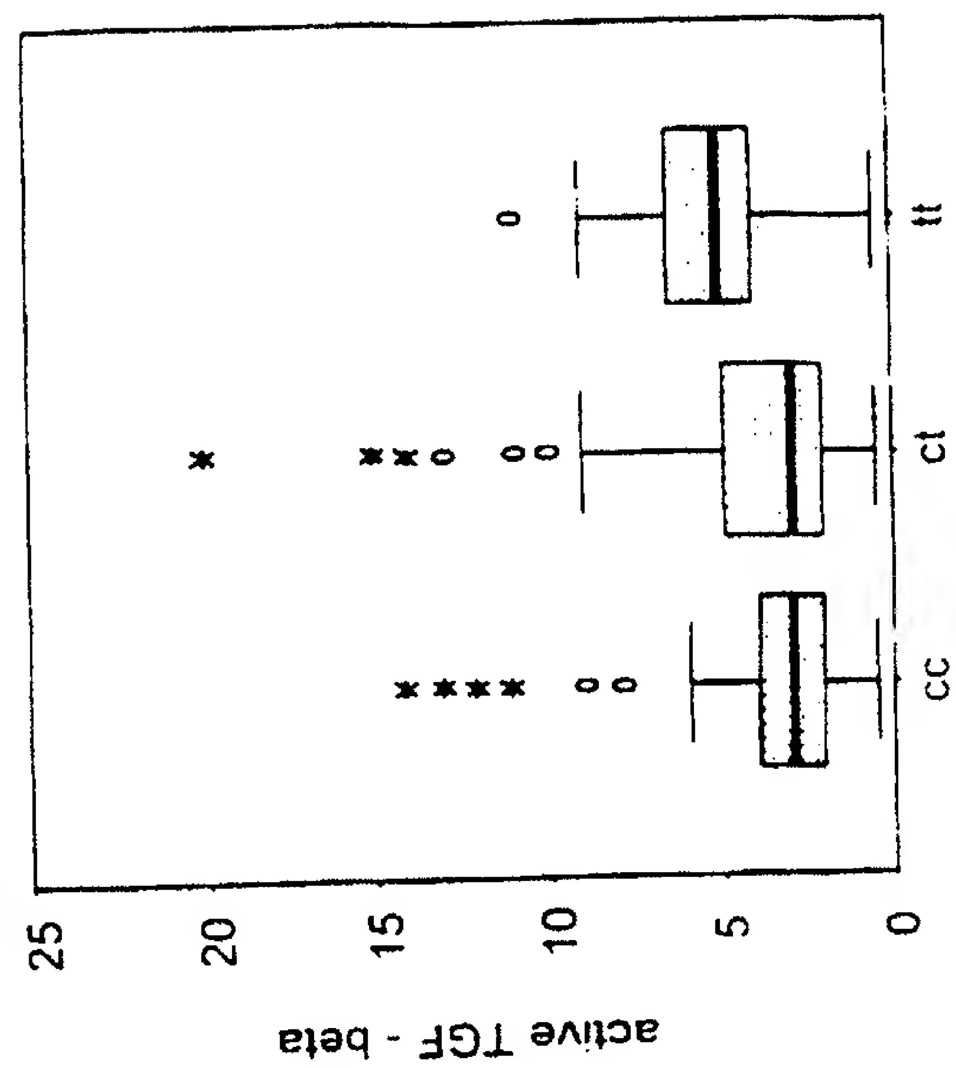


FIG. 4D



INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 97/00425

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 15334 A (CALIFORNIA PACIFIC MED CENTER) 8 June 1995 see claims and abstract ---	36
X	WO 93 01315 A (BLOOD CENTER OF SOUTHEASTERN W) 21 January 1993 see claims and abstract ---	38
X	WO 94 03633 A (GARVAN INST MED RES ;MORRISON NIGEL ALEXANDER (AU); EISMAN JOHN AL) 17 February 1994 see claims and abstract ---	35
X	WO 86 06102 A (BIOTECH RES PARTNERS LTD) 23 October 1986 see abstract --- -/--	37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 July 1997	23.07.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Müller, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	J. BIOL. CHEM., vol. 264, no. 1, - 5 January 1989 pages 402-408, XP002035025 KIM S.-J. ET AL.,: "Characetrization of the promoter region of the human transforming growth factor-beta 1 gene" cited in the application see the whole document ---	1-16, 30-34, 39,40
A	J. BIOL. CHEM., vol. 264, no. 12, 25 April 1989, pages 7041-7045, XP002035026 KIM S.-J. ET AL.,: "Promoter sequences of the humna transforming growth factor-betal gene responsive to transforming growth factor -betal autoinduction" see the whole document ---	1-16, 30-34, 39,40
A	J. BIOL. CHEM., vol. 265, no. 31, - 5 November 1990 pages 19128-19136, XP002035027 LAFYATIS R. ET AL.,: "Structural and functional chararterization of the transforming growth factor beta 3 promoter" see the whole document -----	1-16, 30-34, 39,40

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 97/00425

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9515334 A	08-06-95	AU 1214995 A	19-06-95
WO 9301315 A	21-01-93	AU 2254792 A	11-02-93
		EP 0593572 A	27-04-94
		JP 6508993 T	13-10-94
WO 9403633 A	17-02-94	AU 673187 B	31-10-96
		AU 4690093 A	03-03-94
		CA 2141300 A	17-02-94
		EP 0652975 A	17-05-95
		JP 8501933 T	05-03-96
		US 5593833 A	14-01-97
WO 8606102 A	23-10-86	US 4801531 A	31-01-89
		AU 598192 B	21-06-90
		AU 5776086 A	05-11-86
		EP 0219530 A	29-04-87
		JP 62502940 T	26-11-87

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
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Date of publication of the amended claims: 27 July 2000 (27.07.00)

(54) Title: POLYMORPHISM IN A TGF-BETA GENE CORRELATED TO OSTEOPOROSIS

(57) Abstract

Methods and apparatus for diagnosis of disease and predisposition to disease are described. In particular, methods for diagnosis of osteoporosis and determination of bone mineral density in individuals are provided as well as treatments and therapies for delaying and preventing the onset of disease in at-risk individuals.

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AMENDED CLAIMS

[received by the International Bureau on 09 May 2000 (09.05.00); original claims 1-27 replaced by new claims 1-23 (3 pages)]

1. A method of diagnosing osteoporosis or predisposition to osteoporosis in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

2. A method according to claim 1 wherein presence of a *BstUI* restriction site in said intron 5 is correlated with increased risk of osteoporosis or predisposition to osteoporosis.

3. A method according to claims 1 and 2, wherein said TGF-β gene is the TGF-β1 gene.

4. A method according any previous claim, comprising amplifying a portion of said individual's TGF-β gene, said portion including at least a part of said intron 5, by using PCR techniques.

5. An isolated DNA molecule comprising the sequence of SEQ ID NO: 1.

6. A kit for diagnosis of osteoporosis or predisposition to osteoporosis comprising PCR primers for use in amplification of intron 5 in a TGF-β gene, and reference means to enable determination of the genotype of said intron 5.

7. A kit according to claim 6 wherein said TGF-β gene is a TGF-β1 gene.

8. A kit according to claims 6 and 7 wherein said PCR primers comprise SEQ ID NO: 2.

9. A kit according to any of claims 6-8 wherein said PCR primers comprise SEQ ID NO:3.

10. A kit according to any of claims 6-9 further comprising the restriction enzyme *BstUI*.

11. A method of osteoporosis therapy comprising:-

screening an individual for a genetic predisposition to osteoporosis; and

if such a predisposition is identified, treating that individual to prevent osteoporosis or to delay onset of osteoporosis, wherein a predisposition to osteoporosis is correlated with a polymorphism in intron 5 of a TGF-β1 gene.

12. A method according to claim 11 in which a predisposition to osteoporosis is correlated with the presence of a *BstUI* restriction site in said intron 5 of a TGF-β1 gene.

13. A method according to claims 11 and 12 in which a predisposition to osteoporosis is correlated with a substitution of a thymidine with a cytosine at position 31 in SEQ ID NO:1.

14. A method of treating an individual predisposed or susceptible to osteoporosis said method comprising:-

(a) determining the genotype of intron 5 in a TGF-β1 gene in said individual in order to identify a risk genotype in said TGF-β1 gene; and

(b) administering to said individual an effective dose of a therapeutic composition suitable to delay, reduce, or prevent osteoporosis in said animal.

15. The method of claim 14 comprising administering to said individual an effective dose of said therapeutic composition selected from a group consisting of steroid hormones, isoflavones, calcium supplements, bisphosphonates, calcitonin, sodium fluoride, parathyroid hormone and calcitriol.

16. A method for predicting response to osteoporosis therapy, comprising diagnosing the genotype of intron 5 in a TGF-ß gene according to the method of claim 1.

17. Use, in the manufacture of means for assessing whether an individual has a predisposition to osteoporosis, of PCR primers adapted to amplify a region of a TGF-β gene, said region including intron 5.

18. Use according to Claim 17 wherein said TGF-β gene is a TGF-β1 gene.

19. A method of determining bone mineral density in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

20. A method of determining risk of fracture in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

21. A method of determining bone turnover in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

22. A method according to any of claims 19-21, wherein identification of a *BstUI* restriction site in said intron 5 is diagnostic of increased risk and absence of said *BstUI* restriction site is diagnostic of reduced risk.

23. A method of diagnosing osteoarthritis and predisposition to osteoarthritis in an individual comprising determining the genotype of intron 5 in a TGF-β1 gene.

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POLYMORPHISM IN A TGF-SS GENE CORRELATED TO OSTEOPORPSIS

This invention relates to diagnostic method and apparatus based upon polymorphism of a TGF-ß Gene. More specifically, this invention relates to a method for diagnosis of pre-disposition to disease by screening for the presence of a polymorphism. The invention also relates to apparatus for screening for the polymorphism. The invention further relates to TGF-ß genes containing a polymorphism and to a probe therefor.

Osteoporosis is a common age-related condition characterised by reduced bone mineral density (BMD), deterioration in skeletal microarchitecture and an increased risk of fragility fracture. One in three caucasian women will experience an osteoporotic fracture during their lifetime and it is estimated that the annual health cost of such fractures amounts to around US$14 billion in the USA alone.

Hormone replacement therapy is an established treatment for osteoporosis and has proved successful in halting further decline in bone density that is characteristic in women suffering from this disease. Hormone replacement therapy is generally not, however, able to bring about a reversal of osteoporosis, that is to say it is not capable of inducing an increase in the bone density of sufferers. The same criticism is made of other known treatments for osteoporosis.

It would, accordingly, be of particular advantage to be able to identify with increased accuracy those individuals having a predisposition or increased susceptibility to osteoporosis. Suitable therapy could then be put into place before the effects of osteoporosis set in.

Genetic factors play an important role in determining bone mineral density

(BMD) in later life, with the genetic influence mediated through effects on both peak mass and on age- and menopause- related bone loss. At the menopause there is an increase in the production and activity of various cytokines and growth factors within the bone microenvironment.

Bone mineral density (BMD) in later life is a strong predictor of subsequent osteoporotic fracture and is determined by both the peak value achieved during skeletal growth and by age- and menopause- related bone loss. Family and twin studies suggest a strong genetic component to the determination of peak bone mass, with 50-85% of the population variance in BMD being attributable to genetic factors. Twin studies in postmenopausal and elderly women also support a persistent and significant genetic influence on bone mass in later life. This may represent either a strong residual effect from the genetic contribution to peak bone mass or an independent genetic effect on the regulation of bone loss. Indirect assessment of bone turnover through biochemical markers suggests a genetic regulation of bone metabolism that may translate into differing effects on bone loss although to date only two twin studies have directly attempted to explore the genetic contribution to age- and menopause- related bone loss with conflicting and uncertain results.

Osteoporosis is a complex disease that is likely to have a polygenic aetiology, and candidate gene analysis has demonstrated that polymorphisms of the vitamin D receptor (VDR) locus the oestrogen receptor (ER) locus and the type I collagen alpha 1 (COL1A1) locus are all potential genetic markers for bone mass and bone loss. WO-A-97/28280 describes two polymorphisms in the promoter region of the TGF-β1 gene that have been shown to influence the amount of TGF-β1 protein in the blood and correlates these polymorphisms with predisposition to a number of disease states including hypertension, cancer and osteoporosis.

The search for further genetic markers for use in diagnosis of disease,

including diagnosis of osteoporosis and predisposition thereto, nevertheless continues.

It is an object of this invention to provide method and apparatus for detecting individuals having a predisposition or susceptibility to osteoporosis. It is a further object of the invention to identify individuals having such a predisposition or susceptibility by identifying those individuals on the basis of genotype. It is another object of the invention to provide a therapy for those individuals. Still further objects of the invention are to provide an isolated gene comprising a polymorphism indicative of predisposition to osteoporosis and probe therefor.

Accordingly, a first aspect of the invention provides a method of diagnosis comprising determining genotype of an intron of a TGF-ß gene.

The method of the invention typically comprises determining whether an individual is homozygous or heterozygous for the gene and a particular polymorphism thereof and thereby determining if the individual possesses a risk polymorphism, the risk polymorphism being correlated with osteoporosis or disposition thereto. The method is conveniently used to screen for an individual at risk of a condition or disease correlated with a polymorphism of this gene. In a preferred embodiment of the invention the TGF-β gene is a TGF-β1 gene and the polymorphism is a T→C polymorphism located in intron 5 of said TGF-β1 gene. However, it is envisaged that other polymorphisms that are located within the same or a different intron may be so correlated with the presence of the intron 5 polymorphism of the invention that the two polymorphisms are in linkage disequilibrium. Thus, diagnosis of disease by determining genotype of the further polymorphism may lead to a similarly reliable diagnosis of osteoporosis or predisposition thereto.

The method of the invention determines whether the individual being tested

has a TGF-ß gene which is identical with the published sequence or whether that individual has a gene which differs from the published sequence, i.e. is a polymorphism of the published sequence. In carrying out the invention, an individual's TGF-ß gene genotype is generally determined by analysis of a section of the gene, rather than by analysis of the entire gene. If the sequence of that section is found to be the same as the corresponding section in the wild type sequence, then that individual is classified as having the wild type gene.

In use of an embodiment of the invention to be described below in further detail, an individual is screened to determine whether he or she possess a TGF-ß gene which is the published sequence or is a polymorphism thereof in which there is a polymorphism in one or more of its introns. In this specific embodiment, the presence of a homozygous polymorphism in an intron of the gene correlates with a predisposition to osteoporosis.

In an example of the invention, described in more detail below, the presence of a *BstUI* restriction site in intron 5 of a TGF-β1 gene is correlated with increased risk of osteoporosis or predisposition to osteoporosis as well as increased bone turnover and reduced BMD. This novel restriction site polymorphism is produced as a result of a T→C substitution introducing the 5'-CG↓CG-3' recognition site for *BstUI*.

Screening is carried out, for example, using PCR primers adapted to amplify a portion of gene in the region of and including the site of the polymorphism It is preferred that the PCR primers are selected so as to amplify a region of the gene that surrounds the region and includes at least six nucleotides on either side. PCR techniques are well known in the art and it would be within the ambit of a person of ordinary skill in this art to identify primers for amplifying a suitable section of the gene. PCR techniques are described for example in EP-A-0200362 and EP-A-0201184.

A second aspect of the invention provides for an isolated DNA fragment comprising all or a part of the sequence of SEQ ID NO:1. The sequence in SEQ ID NO:1 is the last fifty bases of intron 5 of the human TGF-β1 gene including the T→C substitution at position 31 of that sequence. Such a DNA fragment may suitably be inserted into a vector for transformation into prokaryotic or eukaryotic cells. Alternatively, the fragment may serve as a template for production of a nucleic acid probe sequence for use in applications such as northern or southern blotting or RNase protection. Thus the invention provides DNA probes capable of distinguishing between a wild type gene, to which the probe does not bind, and a polymorphism thereof, such as one containing a polymorphism in intron 5 to which the probe does bind.

The invention is of advantage in that by screening for the presence of the polymorphism it is possible to identify individuals likely to have a genetic predisposition to this disease.

A third aspect of the invention provides diagnostic means comprising PCR primers adapted to amplify a region of a TGF-ß gene, preferably a DNA segment comprising intron 5.

Suitable primers are recited in the example below and correspond to SEQ ID NOS: 2 and 3. The invention further provides a diagnostic kit comprising PCR primers for use in amplification of an intron in a TGF-β gene and reference means to enable determination of the genotype, optionally within a container. Such reference means typically include control reactions, a written manual or diagrammatic representations indicating positive and negative results.

The kit of the invention may also comprise an amount of the restriction enzyme *BstUI* enabling PCR amplimers of the intron 5 region of the TGF-β1 gene to be analysed for the presence of the novel T→C polymorphism of

the invention.

Accordingly, a fourth aspect of the invention provides a method of therapy comprising screening an individual for a predisposition to osteoporosis and, if a genetic predisposition is identified, treating that individual to delay or reduce or prevent the osteoporosis, wherein a predisposition to osteoporosis is correlated with a polymorphism in an intron of a TGF-β1 gene.

A suitable treatment to prevent or reduce or delay osteoporosis is hormone replacement therapy. The use of this therapy is well known in the art. According to the invention, hormone replacement therapy can thus be commenced in individuals likely to have a predisposition to osteoporosis but in whom osteoporosis has not yet begun to any significant extent.

It is believed that the use of hormone replacement therapy carries with it a concomitant increased risk of breast cancer. The invention offers the advantage that the increased risk of breast cancer associated with hormone replacement therapy can be accepted only by those women who are known to have a likelihood of predisposition to osteoporosis. In an embodiment of this aspect of the invention, the predisposition of an individual to osteoporosis is assessed by determining whether that individual is homozygous for the wild type TGF-ß gene, is heterozygous for the wild type and the, or is homozygous for the polymorphism - indicating risk of predisposition to osteoporosis.

According to the invention, an individual who is homozygous for the risk polymorphism is classified as being at highest risk.

Another suitable treatment is use of bisphosphonates. Two specific treatments involve using xanthine oxidase inhibitors or substituted benzodiazepines and are described in US-A-5436258 and US-A-5441964,

the contents of which are incorporated herein by reference. Still further treatments will be known to a person of skill in the art. Potential treatments are described, for example, in JP-A-09030977, WO-A-97/06254, JP-A-09025293, WO-A-97/04799, WO-A-97/03060 and JP-A-09012592, the contents of which are incorporated herein by reference. Currently authorised treatments for osteoporosis include the use of oestrogens, with and without progestogen, the use of selective oestrogen receptor modulators, the use of anabolic steroids such as nandrolone, the use of the bisphosphonates alendronate and disodium etidronate. Further treatments suitably include the use of salcatonin, administration of calcium supplements and use of isoflavones or other plant derived steroids.

In pharmaceutical treatment of osteoporosis, all routes of administration are suitable and include but are not limited to oral, injection intravenously, intraperitoneally, intramuscularly and subcutaneously, intranasal and topical administration. Typical dosages and durations of treatment are as described in clinician's textbooks such as British National Formulary, incorporated herein by reference.

Currently, none of the osteoporosis medications that have been approved by the Food and Drug Administration (FDA) for postmenopausal women have been approved for men.

Testosterone replacement therapy may be prescribed for a man with a low testosterone level.

Calcitonin is a medication that slows or stops bone loss and may relieve the pain of fractures in some patients. Calcitonin is approved by the FDA for the treatment of osteoporosis in postmenopausal women. While its effect in men has not been studied, evidence suggests that it may work the same in men as in women. Calcitonin is available as an injection and as a nasal spray. Its use is described in US-A-5440012, incorporated herein by

reference.

Bisphosphonates are a class of drugs that have been shown to help preserve and increase bone density by slowing or stopping bone loss. The FDA has approved the bisphosphonate known as alendronate for the treatment of postmenopausal osteoporosis in women; it is currently being studied for treatment of osteoporosis in men. There are other bisphosphonates under development - and in fact etidronate has been approved, though only outside the USA.

Sodium fluoride has recently been recommended for approval by an FDA committee. Parathyroid hormone, calcitriol, and others are investigational drugs. It will be some time before research findings are available on these preparations.

Decrease in bone mineral density can also be slowed by taken calcium supplements, and some suggested levels are 1,000 mg of calcium a day for women on oestrogen replacement therapy and 1,500 mg of calcium daily for women not receiving oestrogen therapy.

Thus, a range of treatments for those suffering or predisposed to osteoporosis are known and all are believed suitable for use in combination with its diagnosis according to the present invention.

Optionally, the assessment of an individual's risk factor is calculated by reference both to the presence of a TGF-ß gene polymorphism and also to other known genetic or physiological or dietary or other indications. The invention in this way provides further information on which measurement of an individual's risk can be based.

The invention thus also provides a method of identifying, and optionally treating, an individual predisposed or susceptible to osteoporosis, said

method comprising determining genotype of a first gene in said individual, wherein genotype of said first gene is correlated with genotype of a TGF-ß gene in said individual.

The invention further again provides a method of predicting response to osteoporosis therapy, comprising diagnosing genotype of a TGF-ß gene, in accordance with the first aspect of the invention.

This latter aspect of the invention thus enables informed choice of therapy, including choice of type of therapy and choice of amount or strength of therapeutic agent, to be made for a given individual predisposed to osteoporosis. Moreover, for a given individual already suffering from osteoporosis, the invention enables an assessment of whether the currently prescribed therapy is likely to be effective in treating the disease or if an alternative therapy regime will be more successful. In a specific embodiment of the invention, diagnosis of a risk polymorphism in a TGF-ß gene indicates that hormone replacement therapy, or an equivalent, is likely to be effective. More specifically, possessing two copies of the risk polymorphism indicates an increased level of therapy is likely to be appropriate.

Bone mineral density (BMD) in later life is a major determinant of osteoporotic fracture risk and has been shown to be under strong genetic influence. Segregation analysis within families and data from twin studies has suggested that this genetic effect on BMD is probably mediated by a number of genes each having small individual effects. Transforming growth factor ß (TGF-ß) is an important regulatory cytokine and is found in high concentrations in the bone matrix. TGF-ß is therefore a plausible candidate for the genetic regulation of BMD. In total 911 DZ pairs and 386 MZ pairs (age range 18-76 years) were studied, with measurements of BMD using DXA and calcaneal ultrasound. In accordance with the present invention, a novel T→C polymorphism was identified in intron 5 with an

allele frequency of 0.25 within the DZ subjects. Comparison of the variance in femoral neck BMD between the MZ and DZ twins showed a heritability of 62% at this site. BMD at the femoral neck was 5% lower in subjects homozygous for the presence of the TGF-ß polymorphism when compared to the other two genotype groups. No effect was seen at the lumbar spine, ultradistal radius, or with ultrasound measurements. Results were unaffected after adjustment for potential confounders. Linkage analysis within the DZ twin pairs confirmed the significance of this polymorphism on hip BMD.

BMD is one of the strongest predictors of fracture risk (Cummings et al. (1993)) and a large number of twin and family studies have suggested a strong genetic influence on this trait, with up to 85% of the population variance in BMD being attributable to genetic factors (Seeman et al.(1989); Arden et al. (1996); Jouanny et al. (1995).

Normal skeletal morphogenesis is dependent on a complex interaction between osteoblasts, osteoclasts, and local growth factors. During growth and development the processes of osteoblastic bone formation and osteoclastic bone resorption appear coupled, thereby maintaining skeletal integrity and preserving bone mass and shape. Non-invasive measures of bone turnover have shown that after the menopause there is an increase in bone resorption, leading to a loss of bone and subsequent development of osteoporosis. Several studies have also shown that in women with osteoporotic fracture the remodelling balance is more negative when compared to age-matched women with no fracture history. It is believed that this postmenopausal increase in bone resorption in the oestrogen deficient state is mediated in part by cytokines (interleukins 1 and 6, tumour necrosis factor and various growth factors (transforming growth factor, insulin-like growth factors). Twin studies have also suggested that there is a genetic influence on the general process of bone turnover in pre- and post-menopausal women, with higher correlations for biochemical

markers of both bone formation and resorption seen in identical compared to non-identical twins (Kelly et al.(1991); Garnero et al.(1996)).

Transforming growth factor ß (TGF-ß) is synthesised by osteoblasts and osteoclasts in vivo and has three isoforms. High concentrations of all these isoforms can be extracted from the mineralised bone matrix, and although TGF-ß is found in a variety of tissues, the concentration of TGF-ß appears to be highest in the bone matrix. Osteoblasts produce TGF-ß largely as a matrix-bound latent complex composed of 390 amino acids. It is only released during bone resorption, with subsequent activation in the acidic environment below the ruffled border of the resorbing osteoclast. This suggests that TGF-ß may play a central regulatory role in the coupling that exists between bone formation and resorption. Active TGF-ß is formed of two identical disulphide-linked polypeptide chains consisting of the 112 amino acids from the C-terminal part of the precursor protein. The TGF-ß gene maps to chromosome 19q13 and contains 7 exons. The active component of TGF-ß is encoded by part of exon 5, exon 6, and part of exon 7. The TGF-ß gene may therefore be an important candidate gene for the development of osteoporosis, with variation at this locus being associated with differences in BMD and therefore risk of fracture.

A further aspect of the invention provides for a method of diagnosing osteoarthritis and predisposition to osteoarthritis in an individual comprising determining the genotype of intron 5 in a TGF-β1 gene, wherein absence of a *BstUI* restriction site is correlated with risk of osteoarthritis.

It is known that predisposition to osteoarthritis and osteoporosis may be linked in that the process of bone deposition seen in osteoarthritis is under the control of similar signalling factors to those that control the process of bone resorption, as seen in osteoporosis. The activities of osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) are to an extent controlled by the same group cytokines including TGF-β. Accordingly, a

further aspect of the present invention provides for a method of diagnosing osteoarthritis and predisposition to osteoarthritis in an individual comprising determining the genotype of intron 5 in a TGF-β1 gene. The risk of disease or predisposition thereto is inversely related to risk of or predisposition to osteoporosis, thus for example absence of a *BstUI* restriction site is correlated with risk of osteoarthritis, and mutatis mutandis for all other embodiments of the invention.

The present invention is now illustrated by way of the following example.

Example

Methods

Subjects

The subjects studied were Caucasian, female monozygous (MZ) and dizygous (DZ) twins (age range 18-76 years) recruited after national media campaigns. All subjects were healthy and did not suffer from diseases specifically affecting bone, and were broadly representative of the normal United Kingdom population as previously described. Twins completed a nurse-administered questionnaire detailing medical, obstetric and gynaecological histories, full drug histories, dietary calcium assessment, exercise levels, smoking status, and alcohol intake. Twin zygosity was determined by questionnaire and in doubtful cases this was confirmed with multiplex DNA fingerprinting.

Measurements

BMD was measured at the lumbar spine (L1-4), non-dominant hip (femoral neck, total hip), and non-dominant ultradistal radius using DXA on a Hologic QDR-2000. Reproducibility as assessed by the coefficient of variation (CV%) from duplicate measures in healthy volunteers was between 0.8% and 1.6% at the skeletal sites measured. Subjects were

classified as having osteoporosis according to the World Health Organisation diagnostic criteria if their BMD measurement was 2.5 standard deviations (SD) below the mean peak young adult value (i.e. T –score < -2.5).

Calcaneal ultrasound was measured using a McHue Cuba Clinical scanner. This produced two output variables: broadband ultrasound attenuation (BUA) and velocity of sound (VOS). Reproducibility as assessed by the CV% in duplicate measures on 30 subjects was 2.5% (BUA) and 0.44% (VOS).

Polymorphism identification

DNA was prepared for each subject from peripheral blood leucocytes using a standard phenol extraction method. Common single nucleotide polymorphisms (SNPs) in the TGF-ß gene were detected by sequence analysis of 24 unrelated dizygotic (DZ) individuals and comparison made with the published sequence (Accession no. Y00112). This strategy was chosen to identify polymorphisms with an allele frequency of at least 0.1 within the study group. Oligonucleotide primer pairs were designed to cover the TGF-ß1 coding regions, and the promoter and 5'-untranslated region up to position 1363. Following amplification by the polymerase chain reaction (PCR) the products were purified with the Advanced Genetic Technologies Corp 96 well PCR purification system and sequenced using PE Applied Biosystems dRhodamine Terminator cycle sequencing kit. The sequencing reactions were analyzed on an ABI 377 DNA sequencer.

Restriction enzyme digests

Polymorphism screening within the DZ group was performed using PCR-restriction fragment length polymorphism (RFLP) based methods with restriction enzyme digest.

BstUI PCR-RFLP analysis of the intron 5 SNP

PCR amplification was performed using the PCR primers SEQ ID NOS: 2 and 3. Reactions were performed in 25μl with the following composition: 0.3μM primers, 0.2mM dNTPs, 1mM MgCL2, 1X Taqgold buffer, 1.25 units Taqgold (P.E. Applied Biosystems) and 50ng genomic DNA. Thermocycling was performed on a MJ Research DNA Engine Tetrad PTC-225 thermal cycler using the following conditions: 95°C for 14 minutes, 35 cycles of 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 30 seconds, followed by final extension of 72°C for 10 minutes. The 230 bp PCR product was digested with 3 units of *BstUI* (New England Biolabs) at 60°C for 2 hours producing polymorphic fragments of 202 and 28 bp. Products were analysed by agarose gel electrophoresis with size determination after transillumination under ultra-violet light. Alleles were coded as A1 = presence of restriction site, and A0 = absence of site.

Statistical analysis

Differences in the mean value in each variable for the three TGF-ß intron 5 genotypes (A0A0, A0A1 and A1A1) were tested using generalised estimating equations (GEE)(Zeger et al. (1986)). This method takes into account the dependence of measurements within twin pairs in estimating the significance of the differences. The GEE models were extended to include potential confounding variables (age, menopausal status). Significant GEE results were followed up using a quantitative genetic modelling approach in which additional phenotypic data on MZ twins were used. A model was specified which provided estimates for the genetic and environmental variance components and estimates for the means of each possible genotype (A0A0, A0A1 and A1A1). This enabled us to estimate the percentage of the genetic variation in the phenotype that could be attributed to the TGF-ß polymorphism. Model fitting was performed using Mx. Parameters were estimated by normal-theory maximum-likelihood,

where the model were fitted to the raw data. Linkage analysis within the DZ twins was also analysed using the MAPMAKER/SIBS programme (version 1.0). This calculates the maximum likelihood sharing probabilities for each DZ pair and as parental information was not available this utilised estimated allele frequencies from within the total DZ group. Evidence for linkage of the polymorphism to the trait was taken as a nominal lod score ≥ 1.00 or a nominal P value ≤ 0.05.

Results

Sequence analysis of 24 unrelated individuals identified a novel polymorphism in the TGF-ß gene. The novel polymorphism was a T→C substitution in intron 5, 20bp upstream of exon 6. This substitution in intron 5 introduces a *BstUI* site (recognition sequence 5'-CG↓CG-3'), and subsequent screening for this within the whole DZ cohort using the *BstUI* PCR-RFLP showed the allele frequencies to be 0.75 and 0.25, with the genotype distributions being in Hardy Weinberg equilibrium.

In total, data was available on 911 DZ and 386 MZ twin pairs. Intron 5 genotype results were available on 1664 of the DZ subjects. Reasons for absence of genetic results included inadequate DNA extraction or failure of the PCR-RFLP assay. No significant differences were observed, however, between subjects with and without TGF-ß genotype results. Characteristics of the study population are shown in Table 1. In comparison with the DZ twins, the MZ twins were on average slightly older by 2 years and had a higher percentage of women who were postmenopausal. Within the DZ group there were no differences in age, height, weight, smoking status or hormone replacement (HRT) use between the three TGF-ß intron 5 genotype groups. There were however, small differences in the proportion of postmenopausal women (Table 1).

The TGF-ß intron 5 genotype A1A1 was associated with hip BMD when compared to the other 2 genotypes, with a 5% reduction in femoral neck BMD and a 3.8% reduction in total hip BMD (Table 2). These findings were unaltered after adjustment for the small difference observed in menopausal status. These results at the hip, suggest a recessive pattern of risk associated with carriage of the rarer A1 TGF-ß intron 5 allele. No genotypic association was seen at the lumbar spine, ultradistal radius or in the calcaneal ultrasound parameters of BUA and VOS. The prevalence of clinically defined osteoporosis (T-score <-2.5) at the femoral neck in the

genotype group A1A1 was 18% compared to 10% in the A0A0 and A0A1 groups. This demonstrates a 70% increased risk for a subject with the A1A1 genotype having femoral neck osteoporosis when compared to the other 2 genotypes, with an odds ratio (95% confidence interval) of 1.71 (0.95, 3.07), P = 0.07.

Having demonstrated an association between TGF-ß intron 5 genotype and hip BMD, twin modelling and variance component analysis was utilised to estimate the proportion of the genetic variance explained by this polymorphism. This analysis confirmed previous studies showing high heritabilities for BMD and ultrasound parameters at the various skeletal sites. At the femoral neck the estimated heritability was 0.62, with the proportion of the population genetic variance explained by the intron 5 TGF-ß polymorphism at this site being 0.60%.

The results of the single point linkage analysis are shown in Table 3. These demonstrate linkage between the intron 5 polymorphism and femoral neck BMD. For the measurement of BUA the nominal P value approaches 0.05 although the lod score does not exceed 1.00.

Thus, in accordance with the present invention, a novel T→C polymorphism in a TGF-ß intron, specifically intron 5 in an embodiment of the invention, of the human TGF-ß gene. Our data demonstrate both association and linkage between this polymorphism and hip BMD in a large group of unselected, normal female twins. The polymorphic C allele was present at a relatively high allelic frequency in the female population (0.25), and there appeared to be a recessive pattern of risk associated with this allelic variant. Hip BMD was 3-5% lower in women who were homozygous for the carriage of the polymorphic allele, when compared to women who were either heterozygous or homozygous for the commoner allele. Women who were homozygous for the polymorphism also had a 70% increased risk of having osteoporosis at the femoral neck in comparison to the other two

genotypes.

These cross-sectional data indicate that subjects who are homozygous for an intronic polymorphism of the TGF-ß gene have reduced BMD at the femoral neck and total hip. The observation that these findings were confined to the hip (and predominantly at the femoral neck), rather than being seen at the spine and distal radius suggest a site-specific association. The absence of any genotype association with bone ultrasound measures also suggests that the TGF-ß intron 5 polymorphism has a predominant effect on BMD rather than on bone quality or structure. Although the proportion of population variance in BMD attributable to this polymorphism appears low, this reflects the recessive risk associated with this locus as only approximately 6% of the population would be expected to be of the genotype A1A1. Our data indicate, however that these subjects have a 70% increased risk of having osteoporosis and therefore face significantly increased risk of fracture. TGF-ß genotype therefore identifies an at risk sub-group of women who would benefit from targeted intervention. This is also illustrated by recent findings from a large population study examining the relationship between a polymorphic variant in the promoter region of the type I collagen 1α gene and osteoporosis. In this study the polymorphic allele was associated with a 2-fold increased risk of fracture despite only explaining 0.3 to 0.4% of the population variance in BMD.

There is currently a large amount of data suggesting that TGF-ß may play a central role in the regulation of BMD and bone turnover. In vivo studies have shown that local injection of TGF-ß under the periosteum stimulates cartilage and bone formation (Noda et al. (1989)) and that systemic injection of TGF-ß2 also leads to a generalised increase in osteoblastic activity. In vitro, TGF-ß induces extracellular matrix secretion by osteoblasts, inhibits matrix mineralisation, and modulates osteoprogenitor cell proliferation. The rate of bone formation is altered in the TGF-ß knockout mouse (Geiser et al. (1996)) and administration of TGF-ß corrects

the bone density deficiency in elderly mice with osteoporosis. Oestrogen action on bone also appears to be mediated via effects on TGF-ß and induction of osteoclast apoptosis. Allelic variation at the TGF-ß locus may therefore be important in determining the therapeutic response of the oestrogen and selective oestrogen receptor modulators on bone and other tissues.

The functional significance of our findings on TGF-ß activity is uncertain, although as the polymorphism is 20 bp upstream of exon 6 it could have some influence on the active component of the TGF-ß protein. The lack of coding sequence variation identified in this study and others suggests that the amino acid sequence of the active form of TGF-ß is highly conserved with strong selective pressures against variant proteins. It has been reported that up to 15% of human diseases are caused by point-sequence variation in splice regions resulting in either exon skipping or cryptic splicing and although the intron 5 polymorphism is not located in a splice donor or acceptor site it could affect a branch point. The intronic polymorphic sequence change may also affect messenger RNA stability and further studies will be required to examine these possibilities. If this polymorphism is not functional, then these findings may suggest that the TGF-ß polymorphism is actually in linkage disequilibrium with a novel disease locus mapping to this chromosomal region. The finding of a positive linkage result at this locus with hip BMD would also implicate this chromosomal region and multipoint linkage analysis will be required to refine the location of the putative disease locus.

The invention thus provides method and apparatus for diagnosis of osteoporosis or predisposition thereto.

Table 1

Mean (±SD) characteristics of female twin subjects

Variable	MZ total (n=724)	DZ total (n=1758)	DZ subjects according to TGF-β1 intron 5 genotype		
			A0A0 (n = 848)	A0A1 (n = 609)	A1A1 (n = 90)
Age (yrs)	50.1 (13.4)	47.5 (11.3)	47.6 (11.0)	47.5 (11.9)	49.1 (9.6)
Height (cm)	162 (6)	163 (6)	162 (6)	163 (6)	162 (6)
Weight (kg)	63.9 (10.7)	65.9 (12.3)	66.0 (12.1)	65.4 (12.1)	65.4 (12.7)
Subjects postmenopausal (%)	67	54	51	56	62
Subjects ever smoking (%)	44	48	45	49	51
Subjects ever use of HRT (%)	30	30	28	31	31

Table 2

Mean (±SD) BMD at lumbar spine, hip and forearm, and calcaneal ultrasound measurements in DZ subjects according to their TGF-β1 intron 5 genotype

Variable	TGF-β1 Intron 5 Genotype		
	A0A0 (n = 848)	A0A1 (n = 609)	A1A1 (n = 90)
Lumbar spine BMD (g/cm²)	0.998 (0.147)	1.011 (0.149)	1.005 (0.146)
Femoral neck BMD (g/cm²)	0.812 (0.131)	0.810 (0.130)	0.770 (0.112)*
Total hip BMD (g/cm²)	0.924 (0.132)	0.925 (0.131)	0.889 (0.117)‡
Ultradistal radius BMD (g/cm²)	0.455 (0.068)	0.458 (0.069)	0.451 (0.068)
VOS (m/sec)	1659 (52)	1660 (52)	1661 (51)
BUA (dB/MHz/cm)	78 (19)	78 (19)	76 (17)

* A1A1 vs A0A0, P = 0.005; A1A1 vs A0A1, P = 0.04

‡ A1A1 vs A0A1, P = 0.05

Table 3

Single point lod scores for TGF-β1 intron 5 genotype and BMD/Ultrasound measures in DZ twin pairs.

Variable	TGF-β1 Intron 5	
	Lod score	Nominal P-value
Lumbar spine BMD (g/cm^2)	0.365	0.195
Femoral neck BMD (g/cm^2)	1.091	0.025
Total hip BMD (g/cm^2)	0.479	0.138
Ultradistal radius BMD (g/cm^2)	0.570	0.105
VOS (m/sec)	0.159	0.392
BUA (dB/MHz/cm)	0.785	0.057

References:

Arden NK, Baker J, Hogg C, Baan K, Spector TD 1996. The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins. J Bone Miner Res 11: 530-534.

Cummings SR, Black DM, Nevitt MC, Browner W, Cauley J, Ensrud K, Genant HK, Palermo L, Scott J, Vogt TM 1993. Bone density at various sites for prediction of hip fractures. The study of osteoporotic fractures research group. Lancet 341: 72-75.

Garnero P, Arden NK, Griffiths GO, Delmas PD, Spector TD 1996 Genetic influences on bone turnover in postmenopausal twins. J Clin Endocrinol Metab 81: 140-146.

Geiser AG, Helvering LM, Zeng QQ, Sato M 1996 Bone growth in the TGF-β1 knockout mouse. J Bone Miner Res 11: S378. (Abstr)

Jouanny P, Guillemin F, Kuntz C, Jeandel C, Pourel J 1995. Environmental and genetic factors affecting bone mass: similarity of bone density among members of healthy families. Arthritis Rheum 38: 61-67.

Kelly PJ, Hopper JL, Macaskill GT, Pocock NA, Sambrook PN, Eisman JA 1991 Genetic factors in bone turnover. J Clin Endocrinol Metab 72: 808-813.

Noda M, Camilliere JJ 1989 In vivo stimulation of bone formation by transforming growth factor β. Endocrinology 124: 2991-2994.

Seeman E, Hopper JL, Bach L, Cooper ME, Parkinson E, McKay J, Jerums G 1989. Reduced bone mass in daughters of women with osteoporosis. N Engl J Med 320: 554-558.

Zeger SL, Liang KY 1986 Longitudinal data analysis for discrete and continuous outcomes. Biometrics 42: 121-30

Claims:

1. A method of diagnosing osteoporosis or predisposition to osteoporosis in an individual comprising determining the genotype of an intron in a TGF-β gene.

2. A method according to Claim 1 wherein said intron is intron 5 of a TGF-β gene.

3. A method according to Claim 2 wherein presence of a *BstUI* restriction site in said intron 5 is correlated with increased risk of osteoporosis or predisposition to osteoporosis.

4. A method according to any previous claim wherein said TGF-β gene is the TGF-β1 gene.

5. A method according to the previous claim comprising amplifying a portion of said individual's TGF-β gene, said portion including at least one intron, by using PCR techniques.

6. An isolated DNA molecule comprising the sequence of SEQ ID NO: 1.

7. A kit for diagnosis of osteoporosis or predisposition to osteoporosis comprising PCR primers for use in amplification of an intron in a TGF-β gene, and reference means to enable determination of the genotype of said intron.

8. A kit according to Claim 7 wherein said intron is intron 5 of a TGF-β gene.

9. A kit according to Claim 7 or 8 wherein said TGF-β gene is a TGF-β1 gene.

10. A kit according to any of Claims 7-9 wherein said PCR primers comprise SEQ ID NO: 2.

11. A kit according to any of Claims 7-10 wherein said PCR primers comprise SEQ ID NO:3.

12. A kit according to any of Claims 7-10 further comprising the restriction enzyme *BstUI*.

13. A method of osteoporosis therapy comprising:-

screening an individual for a genetic predisposition to osteoporosis; and

if such a predisposition is identified, treating that individual to prevent osteoporosis or to delay onset of osteoporosis, wherein a predisposition to osteoporosis is correlated with a polymorphism in an intron of a TGF-β1 gene.

14. A method according to Claim 12 wherein said polymorphism is in intron 5 of the TGF-β1 gene.

15. A method according to Claim 14 in which a predisposition to osteoporosis is correlated with the presence of a *BstUI* restriction site in intron 5 of a TGF-β1 gene.

16. A method according to any of Claims 13-15 in which a predisposition to osteoporosis is correlated with a substitution of a thymidine with a cytosine at position 31 in SEQ ID NO:1.

17. A method of treating an individual predisposed or susceptible to osteoporosis said method comprising:-

(a) determining the genotype of an intron in a TGF-β1 gene in said individual in order to identify a risk genotype in said TGF-β1 gene; and

(b) administering to said individual an effective dose of a therapeutic composition suitable to delay, reduce, or prevent osteoporosis in said animal.

18. The method of Claim 17 comprising administering to said individual an effective dose of said therapeutic composition selected from a group consisting of steroid hormones, isoflavones, calcium supplements, bisphosphonates, calcitonin, sodium fluoride, parathyroid hormone and calcitriol.

19. A method for predicting response to osteoporosis therapy, comprising diagnosing genotype of an intron in a TGF-ß gene according to the method of claim 1.

20. Use, in the manufacture of means for assessing whether an individual has a predisposition to osteoporosis, of PCR primers adapted to amplify a region of a TGF-β gene, said region including intron 5.

21. Use according to Claim 20 wherein said TGF-β gene is a TGF-β1 gene.

22. A method of determining bone mineral density in an individual comprising determining the genotype of an intron in a TGF-β gene.

23. A method of determining risk of fracture in an individual comprising determining the genotype of an intron in an TGF-β gene.

24. A method of determining bone turnover in an individual comprising determining the genotype of an intron in a TGF-β gene.

25. A method according to any of claims 22-24 wherein said intron is intron 5 of a TGF-β1 gene.

26. A method according to Claim 25 wherein identification of a *BstUI* restriction site in said intron 5 is diagnostic of increased risk and absence of said *BstUI* restriction site is diagnostic of reduced risk.

27. A method of diagnosing osteoarthritis and predisposition to osteoarthritis in an individual comprising determining the genotype of intron 5 in a TGF-β1 gene.

SEQUENCE LISTING

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SPECTOR, TIMOTHY D
KEEN, RICHARD W
GIBSON, FERNANDO
MOLLOY, HELEN R

<120> DIAGNOSTIC METHOD AND APPARATUS BASED ON POLYMORPHISM IN A TGF-BETA GENE

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03446

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 A61P19/10 A61K38/23 A61K38/29 A61K33/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LANGDAHL B L ET AL: "A sequence variation: 713-8delC in the transforming growth factor-beta 1 gene has a higher prevalence in osteoporotic women than in normal women and is associated with very low bone mass in osteoporotic women and increased bone turnover in both osteoporotic and normal women" BONE, vol. 20, no. 3, 1997, pages 289-294, XP002076919 the whole document ---	1,4,5,7, 9,19, 22-24

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 February 2000	09/03/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03446

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YAMADA Y ET AL: "Association of a polymorphism of the transforming growth factor -betal gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women." JOURNAL OF BONE AND MINERAL RESEARCH, (1998 OCT) 13 (10) 1569-76. , XP000877167 the whole document ---	1,4,5,7, 9,19, 22-24
A	EP 0 200 341 A (GENENTECH INC) 5 November 1986 (1986-11-05) figure 2 ---	6
A	EP 0 629 697 A (LILLY CO ELI) 21 December 1994 (1994-12-21) ---	
A	WO 97 28280 A (GRAINGER DAVID JOHN ;SPECTOR TIMOTHY DAVID (GB); HEATHCOTE KIRSTEN) 7 August 1997 (1997-08-07) cited in the application ---	
A	DERYNCK R ET AL: "Intron-exon structure of the human transforming growth factor-beta precursor gene" NUCLEIC ACIDS RESEARCH, XP002076920 ---	
E	EP 0 955 378 A (LANGDAHL BENTE LOMHOLT) 10 November 1999 (1999-11-10) the whole document -----	1,4,5,7, 9,19, 22-24

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/03446

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-18
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1 (iv) PCT - Method for treatment of the human or animal body by therapy

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant. this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/03446

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
EP 0200341	A	05-11-1986	AT	99737	T	15-01-1994
			DE	3689495	D	17-02-1994
			DE	3689495	T	01-06-1994
			DK	127786	A	23-09-1986
			IE	61119	B	05-10-1994
			IL	78197	A	18-07-1991
			JP	1937515	C	09-06-1995
			JP	6059230	B	10-08-1994
			JP	61219395	A	29-09-1986
			JP	2043467	C	09-04-1996
			JP	6169777	A	21-06-1994
			JP	7063378	B	12-07-1995
			US	4886747	A	12-12-1989
			US	5482851	A	09-01-1996
			US	5168051	A	01-12-1992
			US	5801231	A	01-09-1998
			US	5284763	A	08-02-1994
EP 0629697	A	21-12-1994	US	5445941	A	29-08-1995
			AU	2871097	A	25-09-1997
			AU	677319	B	17-04-1997
			AU	6470194	A	22-12-1994
			BR	9402480	A	25-01-1995
			CA	2126294	A	22-12-1994
			CN	1102437	A	10-05-1995
			CZ	9401475	A	14-06-1995
			FI	942958	A	22-12-1994
			HU	70326	A	28-09-1995
			IL	109990	A	20-06-1999
			JP	7184661	A	25-07-1995
			NO	942313	A	22-12-1994
			NZ	286125	A	24-11-1997
			PL	303915	A	09-01-1995
			ZA	9404160	A	13-12-1995
WO 9728280	A	07-08-1997	AU	2304697	A	22-08-1997
			CA	2243191	A	07-08-1997
			EP	0889972	A	13-01-1999
			US	5998137	A	07-12-1999
EP 0955378	A	10-11-1999	NONE			

CORRECTED VERSION*

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(54) Title: POLYMORPHISM IN A TGF-BETA GENE CORRELATED TO OSTEOPOROSIS

(57) Abstract

Methods and apparatus for diagnosis of disease and predisposition to disease are described. In particular, methods for diagnosis of osteoporosis and determination of bone mineral density in individuals are provided as well as treatments and therapies for delaying and preventing the onset of disease in at–risk individuals.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GWS/DC/20648	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB99/03446	International filing date *(day/month/year)* 18/10/1999	Priority date *(day/month/year)* 16/10/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant GEMINI GENOMICS (UK)LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/05/2000	21.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Molina Galan, E Telephone No. +31 70 340 3560

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03446

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1,3-24	as originally filed			
2,2a	as received on	22/12/2000	with letter of	21/12/2000

Claims, No.:

1-23	as amended under Article 19

Sequence listing part of the description, pages:

1/1, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 11-15.

because:

☒ the said international application, or the said claims Nos. 11-15 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-10 and 16-23
	No:	Claims	
Inventive step (IS)	Yes:	Claims	2, 5, 8-10 and 22
	No:	Claims	1, 3, 4, 6, 7, 16-21 and 23
Industrial applicability (IA)	Yes:	Claims	1-10 and 16-23
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

III. Non-establishment of opinion (Continuation)

1 For the assessment of the present claims 11-15 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

2 Claims 11-15 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

V. Reasoned statement (Continuation)

2.1 CITATIONS

Reference is made to the following documents:

D1: Bone 20, 289-294, 3-1997, Langdahl et al.
D2: J. Bone Min. Res. 13, 1569-1576, 10-1998, Yamada et al.

2.2 NOVELTY (Art. 33(2) PCT)

2.2.1 The present application does satisfy the criterion set forth in Article 33(2) PCT and the subject-matter of claims 1-10 and 16-23 is new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

2.3 INVENTIVE STEP (Art. 33(3) PCT)

2.3.1 Document D1 is considered to represent the most relevant state of the art and establishes a correlation between a sequence **variation** (i.e. **polymorphism** or **mutation** with respect to a reference sequence) in intron 5 of the transforming growth factor-beta 1 (TGF-β1) gene and very low bone mass in osteoporotic women. The subject-matter of claim 2 differs in that a different mutation is brought in relationship with increased risk of suffering osteoporosis, the mutation being defined by the presence of a BstUI restriction site in intron 5 of the TGF-β1 gene (result of a T→C substitution).

2.3.2 The problem to be solved by the subject matter of claim 2 may therefore be regarded as providing alternative or additional ways for the prognosis of osteoporosis in an individual. The solution would be the detection of said substitution (by e.g. restriction analysis).

2.3.3 It is known that mutations in the TGF-β1 gene are related to osteoporosis and that their detection have hence a predictive value (see D1 or D2). It is the IPEA's believe however that this knowledge does not make the finding of additional mutations obvious and therefore the isolated DNA sequence (Seq. Id. 1) and methods using it or detecting the mutation contained in it can be considered as involving an inventive step.

2.3.4 The present application does satisfy the criterion set forth in Article 33(3) PCT and the subject-matter of claims 2, 5, 8-10 and 22 does involve an inventive step (Rule 65(1)(2) PCT).

2.3.5 The above reasoning can not be followed however for claims 1, 3, 4, 6, 7, 16-21 and 23 because they express the mere indication that a relevant mutation could be present in intron 5. D1 discloses the presence of mutations (or sequence variations) in introns of TGF-β1 being over represented in patients with osteoporosis. Obviously, the normal population should present this sequence variation in a very low frequency (ideally not at all) if it has to be of any predictive value. The person skilled in the art would therefore be inclined to consider the existence of sequence variations in intronic parts of TGF-β1 being related in some way with a

predisposition to osteoporosis. Moreover, claims 1, 3, 4, 6, 7, 16-21 and 23 do themselves not provide the solution to the problem of diagnosing (predisposition to) osteoporosis as the person skilled in the art would not know what kind of genotyping results would be indicative of the same. The mere indication to genotype a given intron of a gene generally known to be related to osteoporosis can, certainly in the light of D1, not be considered to involve the application of inventive skills. Claims 1, 3, 4, 6, 7, 16-21 and 23 do therefore not involve an inventive step as required by Art. 33(3) PCT (see also box VIII).

VIII. Certain Observations (Continuation)

1 It appears from the description as a whole and in particular from the inventive step reasoning above, that the particular mutation in the TGF-β1 gene is an essential technical feature of the present invention. This essential technical feature is however not present in claims 1, 3, 4, 6, 7, 16-21 and 23. For these reasons the claims lack clarity according to Art. 6 PCT taken in combination with Rule 6.3 (b) PCT (see also PCT Preliminary Examination Guidelines III.4.3).

2 Claims 1, 3, 4, 6, 7, 16-21 and 23 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempts to define the subject-matter in terms of the result to be achieved (diagnosis of osteoporosis) which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result (sequence variations indicative of osteoporosis) are however missing.

3 The specification for an international application should be capable of being understood without reference to any other document (cf PCT Guidelines Ch. II 4.17). To avoid confusion the expressions "hereby incorporated by reference" found in the description are therefore not according to the PCT requirements.

(BMD) in later life, with the genetic influence mediated through effects on both peak mass and on age- and menopause- related bone loss. At the menopause there is an increase in the production and activity of various cytokines and growth factors within the bone microenvironment.

Bone mineral density (BMD) in later life is a strong predictor of subsequent osteoporotic fracture and is determined by both the peak value achieved during skeletal growth and by age- and menopause- related bone loss. Family and twin studies suggest a strong genetic component to the determination of peak bone mass, with 50-85% of the population variance in BMD being attributable to genetic factors. Twin studies in postmenopausal and elderly women also support a persistent and significant genetic influence on bone mass in later life. This may represent either a strong residual effect from the genetic contribution to peak bone mass or an independent genetic effect on the regulation of bone loss. Indirect assessment of bone turnover through biochemical markers suggests a genetic regulation of bone metabolism that may translate into differing effects on bone loss although to date only two twin studies have directly attempted to explore the genetic contribution to age- and menopause- related bone loss with conflicting and uncertain results.

Osteoporosis is a complex disease that is likely to have a polygenic aetiology, and candidate gene analysis has demonstrated that polymorphisms of the vitamin D receptor (VDR) locus the oestrogen receptor (ER) locus and the type I collagen alpha 1 (COL1A1) locus are all potential genetic markers for bone mass and bone loss. WO-A-97/28280 describes two polymorphisms in the promoter region of the TGF-β1 gene that have been shown to influence the amount of TGF-β1 protein in the blood and correlates these polymorphisms with predisposition to a number of disease states including hypertension, cancer and osteoporosis.

~~The search for further genetic markers for use in diagnosis of disease,~~

Langdahl et al. (Bone Vol.20, No.3, 1997: 289-294) describes a sequence variation in an intron of a TGF-Beta 1 gene, the 713-8delC variation. The sequence variation is a single base deletion eight bases upstream of the start of intron 5. The 713-8delC variation is correlated with an increase in bone turnover but in both the non-osteoporotic normal control individuals and the total group of osteoporotic patients bone mass was unaffected by the presence of this variation.

Yamada et al. (J. Bone. Miner. Res. Vol. 13, No.10, 1998:1569-1676) relates to a T→C transition at nucleotide 29 in the signal sequence region of the TGF-Beta 1 gene. Yamada et al. describe the correlation between this variation and susceptibility to osteoporosis in postmenopausal Japanese women.

The search for further genetic markers for use in diagnosis of disease,

Claims:

1. A method of diagnosing osteoporosis or predisposition to osteoporosis in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

2. A method according to claim 1 wherein presence of a *BstUI* restriction site in said intron 5 is correlated with increased risk of osteoporosis or predisposition to osteoporosis.

3. A method according to claims 1 and 2, wherein said TGF-β gene is the TGF-β1 gene.

4. A method according any previous claim, comprising amplifying a portion of said individual's TGF-β gene, said portion including at least a part of said intron 5, by using PCR techniques.

5. An isolated DNA molecule comprising the sequence of SEQ ID NO: 1.

6. A kit for diagnosis of osteoporosis or predisposition to osteoporosis comprising PCR primers for use in amplification of intron 5 in a TGF-β gene, and reference means to enable determination of the genotype of said intron 5.

7. A kit according to claim 6 wherein said TGF-β gene is a TGF-β1 gene.

8. A kit according to claims 6 and 7 wherein said PCR primers comprise SEQ ID NO: 2.

9. A kit according to any of claims 6-8 wherein said PCR primers comprise SEQ ID NO:3.

10. A kit according to any of claims 6-9 further comprising the restriction enzyme *BstUI*.

11. A method of osteoporosis therapy comprising:-

screening an individual for a genetic predisposition to osteoporosis; and

if such a predisposition is identified, treating that individual to prevent osteoporosis or to delay onset of osteoporosis, wherein a predisposition to osteoporosis is correlated with a polymorphism in intron 5 of a TGF-β1 gene.

12. A method according to claim 11 in which a predisposition to osteoporosis is correlated with the presence of a *BstUI* restriction site in said intron 5 of a TGF-β1 gene.

13. A method according to claims 11 and 12 in which a predisposition to osteoporosis is correlated with a substitution of a thymidine with a cytosine at position 31 in SEQ ID NO:1.

14. A method of treating an individual predisposed or susceptible to osteoporosis said method comprising:-

(a) determining the genotype of intron 5 in a TGF-β1 gene in said individual in order to identify a risk genotype in said TGF-β1 gene; and

(b) administering to said individual an effective dose of a therapeutic composition suitable to delay, reduce, or prevent osteoporosis in said animal.

15. The method of claim 14 comprising administering to said individual an effective dose of said therapeutic composition selected from a group consisting of steroid hormones, isoflavones, calcium supplements, bisphosphonates, calcitonin, sodium fluoride, parathyroid hormone and calcitriol.

16. A method for predicting response to osteoporosis therapy, comprising diagnosing the genotype of intron 5 in a TGF-ß gene according to the method of claim 1.

17. Use, in the manufacture of means for assessing whether an individual has a predisposition to osteoporosis, of PCR primers adapted to amplify a region of a TGF-β gene, said region including intron 5.

18. Use according to Claim 17 wherein said TGF-β gene is a TGF-β1 gene.

19. A method of determining bone mineral density in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

20. A method of determining risk of fracture in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

21. A method of determining bone turnover in an individual comprising determining the genotype of intron 5 in a TGF-β gene.

22. A method according to any of claims 19-21, wherein identification of a *BstUI* restriction site in said intron 5 is diagnostic of increased risk and absence of said *BstUI* restriction site is diagnostic of reduced risk.

23. A method of diagnosing osteoarthritis and predisposition to osteoarthritis in an individual comprising determining the genotype of intron 5 in a TGF-β1 gene.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 15 June 2000 (15.06.00)	
International application No. PCT/GB99/03446	**Applicant's or agent's file reference** GWS/DC/20648
International filing date (day/month/year) 18 October 1999 (18.10.99)	**Priority date** (day/month/year) 16 October 1998 (16.10.98)
Applicant SPECTOR, Timothy, David et al	

1. The designated Office is hereby notified of its election made:

   ☒ in the demand filed with the International Preliminary Examining Authority on:

   11 May 2000 (11.05.00)

   ☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was ☐ was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Olivia RANAIVOJAONA Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
MATHYS & SQUIRE
Attn. SCHLICH,GEORGE WILLIAM
100 Gray's Inn Road
London WC1X 8AL
UNITED KINGDOM

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing *(day/month/year)* 09/03/2000	
Applicant's or agent's file reference GWS/DC/20648	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/GB 99/03446	International filing date *(day/month/year)* 18/10/1999
Applicant GEMINI RESEARCH LIMITED et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

   When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

   Where? Directly to the International Bureau of WIPO
   34, chemin des Colombettes
   1211 Geneva 20, Switzerland
   Fascimile No.: (41–22) 740.14.35

   For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   [ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   [ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

   Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

   Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

   Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL–2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Nina Vercio

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference GWS/DC/20648	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/GB 99/ 03446	International filing date *(day/month/year)* 18/10/1999	(Earliest) Priority Date *(day/month/year)* 16/10/1998
Applicant GEMINI RESEARCH LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (see Box II).
4. With regard to the **title**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   POLYMORPHISM IN A TGF-SS GENE CORRELATED TO OSTEOPORPSIS

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. —

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the *International Preliminary Examining Authority*. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 *or, where applicable*, Article 41.

When? Within 2 months from the date of trans[illegible] the international search report or 16 months from the priority date, whichever time limit expires later [illegible]d be noted, however, that the amendments will be considered as having been received on time if the[illegible]eived by the International Bureau after the expiration of the applicable time limit but before the com[illegible] of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a *demand for international preliminary* examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be *confused* with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application-for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

International application No.

PCT/GB 99/03446

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-18
   because they relate to subject matter not required to be searched by this Authority, namely:
   Rule 39.1 (iv) PCT - Method for treatment of the human or animal body by therapy

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03446

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 A61P19/10 A61K38/23 A61K38/29 A61K33/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LANGDAHL B L ET AL: "A sequence variation: 713-8delC in the transforming growth factor-beta 1 gene has a higher prevalence in osteoporotic women than in normal women and is associated with very low bone mass in osteoporotic women and increased bone turnover in both osteoporotic and normal women" BONE, vol. 20, no. 3, 1997, pages 289-294, XP002076919 the whole document --- -/--	1,4,5,7, 9,19, 22-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 February 2000	09/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

1

International Application No
PCT/GB 99/03446

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YAMADA Y ET AL: "Association of a polymorphism of the transforming growth factor -beta1 gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women." JOURNAL OF BONE AND MINERAL RESEARCH, (1998 OCT) 13 (10) 1569-76. , XP000877167 the whole document	1,4,5,7, 9,19, 22-24
A	EP 0 200 341 A (GENENTECH INC) 5 November 1986 (1986-11-05) figure 2	6
A	EP 0 629 697 A (LILLY CO ELI) 21 December 1994 (1994-12-21)	
A	WO 97 28280 A (GRAINGER DAVID JOHN ;SPECTOR TIMOTHY DAVID (GB); HEATHCOTE KIRSTEN) 7 August 1997 (1997-08-07) cited in the application	
A	DERYNCK R ET AL: "Intron-exon structure of the human transforming growth factor-beta precursor gene" NUCLEIC ACIDS RESEARCH, XP002076920	
E	EP 0 955 378 A (LANGDAHL BENTE LOMHOLT) 10 November 1999 (1999-11-10) the whole document	1,4,5,7, 9,19, 22-24

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/03446

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
EP 0200341	A	05-11-1986	AT	99737	T	15-01-1994
			DE	3689495	D	17-02-1994
			DE	3689495	T	01-06-1994
			DK	127786	A	23-09-1986
			IE	61119	B	05-10-1994
			IL	78197	A	18-07-1991
			JP	1937515	C	09-06-1995
			JP	6059230	B	10-08-1994
			JP	61219395	A	29-09-1986
			JP	2043467	C	09-04-1996
			JP	6169777	A	21-06-1994
			JP	7063378	B	12-07-1995
			US	4886747	A	12-12-1989
			US	5482851	A	09-01-1996
			US	5168051	A	01-12-1992
			US	5801231	A	01-09-1998
			US	5284763	A	08-02-1994
EP 0629697	A	21-12-1994	US	5445941	A	29-08-1995
			AU	2871097	A	25-09-1997
			AU	677319	B	17-04-1997
			AU	6470194	A	22-12-1994
			BR	9402480	A	25-01-1995
			CA	2126294	A	22-12-1994
			CN	1102437	A	10-05-1995
			CZ	9401475	A	14-06-1995
			FI	942958	A	22-12-1994
			HU	70326	A	28-09-1995
			IL	109990	A	20-06-1999
			JP	7184661	A	25-07-1995
			NO	942313	A	22-12-1994
			NZ	286125	A	24-11-1997
			PL	303915	A	09-01-1995
			ZA	9404160	A	13-12-1995
WO 9728280	A	07-08-1997	AU	2304697	A	22-08-1997
			CA	2243191	A	07-08-1997
			EP	0889972	A	13-01-1999
			US	5998137	A	07-12-1999
EP 0955378	A	10-11-1999	NONE			

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GWS/DC/20648	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB99/03446	International filing date *(day/month/year)* 18/10/1999	Priority date *(day/month/year)* 16/10/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant GEMINI GENOMICS (UK)LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 11/05/2000	Date of completion of this report 21.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Molina Galan, E Telephone No. +31 70 340 3560

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03446

I. **Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1,3-24	as originally filed			
2,2a	as received on	22/12/2000	with letter of	21/12/2000

Claims, No.:

1-23	as amended under Article 19

Sequence listing part of the description, pages:

1/1, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:

☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 11-15.

because:

☒ the said international application, or the said claims Nos. 11-15 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

International application No. PCT/GB99/03446

Novelty (N)	Yes:	Claims	1-10 and 16-23
	No:	Claims	
Inventive step (IS)	Yes:	Claims	2, 5, 8-10 and 22
	No:	Claims	1, 3, 4, 6, 7, 16-21 and 23
Industrial applicability (IA)	Yes:	Claims	1-10 and 16-23
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

III. **Non-establishment of opinion** (Continuation)

1 For the assessment of the present claims 11-15 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

2 Claims 11-15 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

V. **Reasoned statement** (Continuation)

2.1 CITATIONS

Reference is made to the following documents:

D1: Bone 20, 289-294, 3-1997, Langdahl et al.
D2: J. Bone Min. Res. 13, 1569-1576, 10-1998, Yamada et al.

2.2 NOVELTY (Art. 33(2) PCT)

2.2.1 The present application does satisfy the criterion set forth in Article 33(2) PCT and the subject-matter of claims 1-10 and 16-23 is new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

2.3 INVENTIVE STEP (Art. 33(3) PCT)

2.3.1 Document D1 is considered to represent the most relevant state of the art and establishes a correlation between a sequence **variation** (i.e. **polymorphism** or **mutation** with respect to a reference sequence) in intron 5 of the transforming growth factor-beta 1 (TGF-β1) gene and very low bone mass in osteoporotic women. The subject-matter of claim 2 differs in that a different mutation is brought in relationship with increased risk of suffering osteoporosis, the mutation being defined by the presence of a BstUI restriction site in intron 5 of the TGF-β1 gene (result of a T→C substitution).

2.3.2 The problem to be solved by the subject matter of claim 2 may therefore be regarded as providing alternative or additional ways for the prognosis of osteoporosis in an individual. The solution would be the detection of said substitution (by e.g. restriction analysis).

2.3.3 It is known that mutations in the TGF-β1 gene are related to osteoporosis and that their detection have hence a predictive value (see D1 or D2). It is the IPEA's believe however that this knowledge does not make the finding of additional mutations obvious and therefore the isolated DNA sequence (Seq. Id. 1) and methods using it or detecting the mutation contained in it can be considered as involving an inventive step.

2.3.4 The present application does satisfy the criterion set forth in Article 33(3) PCT and the subject-matter of claims 2, 5, 8-10 and 22 does involve an inventive step (Rule 65(1)(2) PCT).

2.3.5 The above reasoning can not be followed however for claims 1, 3, 4, 6, 7, 16-21 and 23 because they express the mere indication that a relevant mutation could be present in intron 5. D1 discloses the presence of mutations (or sequence variations) in introns of TGF-β1 being over represented in patients with osteoporosis. Obviously, the normal population should present this sequence variation in a very low frequency (ideally not at all) if it has to be of any predictive value. The person skilled in the art would therefore be inclined to consider the existence of sequence variations in intronic parts of TGF-β1 being related in some way with a

predisposition to osteoporosis. Moreover, claims 1, 3, 4, 6, 7, 16-21 and 23 do themselves not provide the solution to the problem of diagnosing (predisposition to) osteoporosis as the person skilled in the art would not know what kind of genotyping results would be indicative of the same. The mere indication to genotype a given intron of a gene generally known to be related to osteoporosis can, certainly in the light of D1, not be considered to involve the application of inventive skills. Claims 1, 3, 4, 6, 7, 16-21 and 23 do therefore not involve an inventive step as required by Art. 33(3) PCT (see also box VIII).

VIII. Certain Observations (Continuation)

1 It appears from the description as a whole and in particular from the inventive step reasoning above, that the particular mutation in the TGF-β1 gene is an essential technical feature of the present invention. This essential technical feature is however not present in claims 1, 3, 4, 6, 7, 16-21 and 23. For these reasons the claims lack clarity according to Art. 6 PCT taken in combination with Rule 6.3 (b) PCT (see also PCT Preliminary Examination Guidelines III.4.3).

2 Claims 1, 3, 4, 6, 7, 16-21 and 23 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempts to define the subject-matter in terms of the result to be achieved (diagnosis of osteoporosis) which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result (sequence variations indicative of osteoporosis) are however missing.

3 The specification for an international application should be capable of being understood without reference to any other document (cf PCT Guidelines Ch. II 4.17). To avoid confusion the expressions "hereby incorporated by reference" found in the description are therefore not according to the PCT requirements.

(BMD) in later life, with the genetic influence mediated through effects on both peak mass and on age- and menopause- related bone loss. At the menopause there is an increase in the production and activity of various cytokines and growth factors within the bone microenvironment.

Bone mineral density (BMD) in later life is a strong predictor of subsequent osteoporotic fracture and is determined by both the peak value achieved during skeletal growth and by age- and menopause- related bone loss. Family and twin studies suggest a strong genetic component to the determination of peak bone mass, with 60-85% of the population variance in BMD being attributable to genetic factors. Twin studies in postmenopausal and elderly women also support a persistent and significant genetic influence on bone mass in later life. This may represent either a strong residual effect from the genetic contribution to peak bone mass or an independent genetic effect on the regulation of bone loss. Indirect assessment of bone turnover through biochemical markers suggests a genetic regulation of bone metabolism that may translate into differing effects on bone loss although to date only two twin studies have directly attempted to explore the genetic contribution to age- and menopause- related bone loss with conflicting and uncertain results.

Osteoporosis is a complex disease that is likely to have a polygenic aetiology, and candidate gene analysis has demonstrated that polymorphisms of the vitamin D receptor (VDR) locus the oestrogen receptor (ER) locus and the type I collagen alpha 1 (COL1A1) locus are all potential genetic markers for bone mass and bone loss. WO-A-97/28280 describes two polymorphisms in the promoter region of the TGF-β1 gene that have been shown to influence the amount of TGF-β1 protein in the blood and correlates these polymorphisms with predisposition to a number of disease states including hypertension, cancer and osteoporosis.

~~The search for further genetic markers for use in diagnosis of disease,~~

Langdahl et al. (Bone Vol.20, No.3, 1997: 289-294) describes a sequence variation in an intron of a TGF-Beta 1 gene, the 713-8delC variation. The sequence variation is a single base deletion eight bases upstream of the start of intron 5. The 713-8delC variation is correlated with an increase in bone turnover but in both the non-osteoporotic normal control individuals and the total group of osteoporotic patients bone mass was unaffected by the presence of this variation.

Yamada et al. (J. Bone. Miner. Res. Vol. 13, No.10, 1998:1569-1676) relates to a T→C transition at nucleotide 29 in the signal sequence region of the TGF-Beta 1 gene. Yamada et al. describe the correlation between this variation and susceptibility to osteoporosis in postmenopausal Japanese women.

The search for further genetic markers for use in diagnosis of disease,

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SCHLICH, George, William
Mathys & Squire
100 Gray's Inn Road
London WC1X 8AL
ROYAUME-UNI

Date of mailing (day/month/year) 30 October 2000 (30.10.00)	
Applicant's or agent's file reference GWS/DC/20648	**IMPORTANT NOTIFICATION**
International application No. PCT/GB99/03446	**International filing date** (day/month/year) 18 October 1999 (18.10.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
GEMINI RESEARCH LIMITED 162 Science Park Milton Road Cambridge CB4 4GH United Kingdom	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
GEMINI GENOMICS (UK) LIMITED 162 Science Park Milton Road Cambridge CB4 4GH United Kingdom	GB	GB
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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Christine Carrié Telephone No.: (41-22) 338.83.38